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Can less yield more? Is reducing nutrient input into the environment compatible with maintaining crop production?

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Plant scientists have long recognized the need to develop crops that absorb and use nutrients more efficiently. Two approaches have been used to increase nutrient use efficiency (NUE) in crop plants. The first involves both traditional breeding and marker-assisted selection in an attempt to identify the genes involved. The second uses novel gene constructs designed to improve specific aspects of NUE. Here, we discuss some recent developments in the genetic manipulation of NUE in crop plants and argue that an improved understanding of the transition between nitrogen assimilation and nitrogen recycling will be important in applying this technology to increasing crop yields. Moreover, we emphasize the need to combine genetic and transgenic approaches to make significant improvements in NUE.

Crop plants have a fundamental dependence on inorganic nitrogenous fertilizers, principally in the form of NO_3^- and NH_4^+ [1]. Approximately 85 million to 90 million metric tons (MMt) of nitrogenous fertilizers are added to soil worldwide annually, up from only 1.3 MMt in 1930 and 10.2 MMt in 1960 [2], and this is predicted to increase to 240 MMt by the year 2050 [3]. It is estimated that 50–70% of the applied nitrogen (N) is lost from the plant–soil system [4]. The ability of plants to capture N from the soil depends on variables including soil type, environment and species [5–7]. Because NO_3^- is soluble and not retained by the soil matrix, excess NO_3^- can leach into the water and also be depleted by microorganisms. Box 1 outlines the inputs and losses of N into the environment as N moves through the soil and plant, eventually being harvested as yield.

It is important to improve the nutrient use efficiency (NUE) of crop plants for two reasons. First, the use of commercial fertilizers is one of the major costs associated with the production of high-yielding crops and, although these costs are substantial for all producers, they are often prohibitive for subsistence farmers. Second, the environmental damage associated with the use of nitrogen-based fertilizers is becoming significant [8]. Nitrogen deposition is also no longer a local problem, with the globalization of nitrogen deposition beginning to have significant consequences for terrestrial ecosystems, particularly as these ecosystems become N saturated [9]. In this article, we focus on molecular approaches that have been designed to enhance N uptake and assimilation in plants by the overexpression of novel transgenes, and discuss their integration with more traditional breeding approaches.

Defining and estimating NUE

Nitrogen is one the most expensive nutrients to supply, therefore one of the objectives of crop improvement programs should be to measure and maximize nutrient use efficiency (NUE). In measuring NUE, several definitions and evaluation methods have been developed over the years (Box 2) [7,10–12]. These definitions differ in a few basic ways. First, measurements of NUE are based on either total biomass (Box 2, Eqns 1,2 in Table I) or grain weight (Box 2, Eqns 3,5,6,8). In addition, several of these definitions look at the efficiency of extracting N from the soil (Box 2, Eqns 4,7). Agronomic efficiency (AE) is the product of physiological efficiency and apparent recovery, and NUEg is the product of uptake efficiency and utilization efficiency (Box 2, Eqns 4,5). Both of these sets of equations reflect the efficiency with which applied nitrogen is used to produce grain yield. Craswell and Godwin's [7] definitions differ from those of Moll *et al.* [12] in that the analysis uses an unfertilized control as the initial starting point for analysis. These equations (Box 2, Eqns 3–8) can also be expanded to include additional factors including physiological ones [7,12]. Clearly, the appropriate way to estimate NUE depends on the crop, its harvest product and whether the researcher wants to analyse specific physiological processes involved in NUE.

Nitrogen uptake, assimilation and transport gene systems

Most plants obtain nitrogen from soil nitrate, which is largely derived from the external supply of inorganic

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Box 1. Sources and fates of nitrogen in plants and the environment

There are many anthropogenic nitrogen (N) inputs into the environment (Figure I). The N input from fertilizers represents 85 million metric tons (MMt) annually, whereas biological fixation of N by legumes and other plants accounts for 40 MMt. Other anthropogenic sources (fossil fuels and habitat destruction) account for a further 90 MMt [9]. Natural sources (soil bacteria, algae, lightning) account for 140 MMt. The key physiological processes for N uptake and conversion to grain yield are highlighted in green boxes. 'Recovery Efficiency' (RE) is defined by Eqns 4,6 in Table I in Box 2; 'Physiological Physiological Efficiency' (PE) is defined by Eqns 5,6 (Box 2).

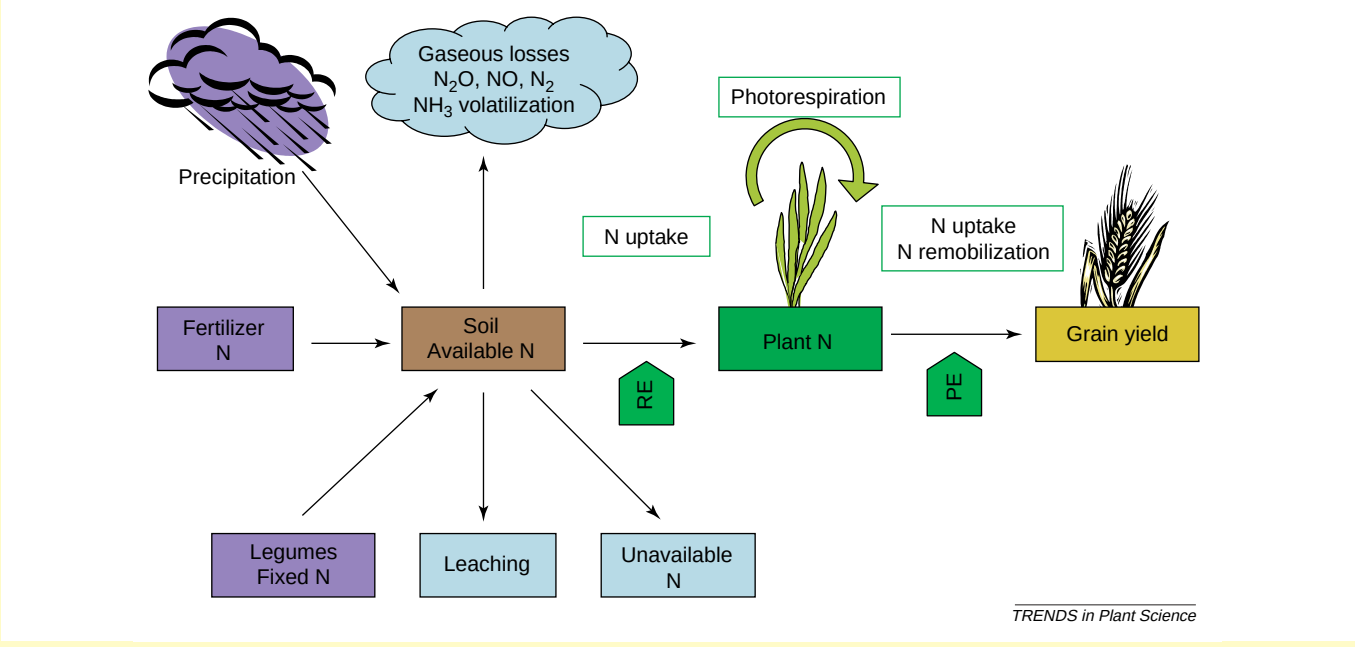

Figure I.

fertilizers, bacterial nitrification or naturally through biological nitrogen fixation (Box 1). Use of nitrogen by plants involves several steps including uptake, assimilation, translocation and remobilization. These steps are outlined below with a discussion of specific attempts to evaluate the overexpression of these gene systems.

Nitrogen transporters

The *Arabidopsis* nitrogen (nitrate) transporter gene *AtNRT1.1* was originally isolated in screens for chlorate resistance and then cloned by T-DNA tagging [13]. This gene is a member of an unusual family of transporters (the PTR family) that is widely distributed in both prokaryotes and eukaryotes, and most members of which function as proton–oligopeptide co-transporters in the plasma membrane [14]. A good deal is known about the regulation of the different nitrate and ammonium transporters in plants [14,15] but, to date, few studies have analysed the specific effects of the overexpression of these genes on plant growth and development (Table 1). Liu *et al.* [16] have shown that overexpression of the dual-affinity nitrate-transporter gene *CHL1* in a *chl* mutant background resulted in recovery of the normal phenotype in terms of nitrate uptake for the constitutive phase but not the induced phase of uptake. Fraisier *et al.* [17] overexpressed the gene that encodes a high-affinity nitrate-transporter in tobacco (*NpNRT2.1*), using both the CaMV 35S and *rolD* promoters. They found that the transgenics showed increased levels of the *NpNRT2.1* transcript and that this was associated with increased nitrate influx under low nitrate conditions. However, the total nitrate contents were similar in the transgenic versus non-transgenic tubers. In summary, the ectopic expression of nitrate transporters has been shown to affect NO_3^- influx but no phenotypic effect on NUE has been seen to date.

The function, cloning and regulation of the ammonium transporters have been well characterized in excellent reviews [18,19]. However, there have been few studies that report the overexpression of these genes in plants and, to date, the consequences of the overexpression of ammonium transporters on NUE or other growth parameters have not been published.

Nitrate and nitrite reductase

Two successive enzymatic steps in the nitrogen assimilation pathway reduce nitrate to ammonia. Nitrate is first converted to nitrite by nitrate reductase (NR) and then nitrite is translocated from the cytoplasm to the chloroplast, where it is reduced by nitrite reductase (NiR) to ammonium. The expression of the NR genes is influenced by several endogenous and environmental factors in plants and is highly regulated at the transcriptional, translational and post-translational levels [20]. In general, mutants devoid of NR activity or transgenic plants underexpressing the NR gene tend to accumulate high levels of nitrate [20,21]. Several studies have been performed on plants in which the NR and NiR genes

Box 2. Definitions and formulae used to describe nutrient use efficiency in plants

Equation 1 (Table I) in essence measures the carbon:nitrogen ratio of the plant. The 'Utilization Index' factors for the absolute amount of biomass produced as well as for the ratio of biomass per unit nitrogen. 'NUEg' is calculated as grain production per unit of N available. There are two primary components of NUEg, which are referred to as 'uptake efficiency' (the efficiency of absorption or uptake of supplied N) and 'usage efficiency' (the efficiency with which the total plant N is used to produce grain) (Eqns 4,5, respectively). For simplicity, fertilizer applied is often substituted for nitrogen supply (designated N_s) and nitrogen in the above ground tissues substituted for total nitrogen (designated N_t). Craswell and Godwin [7] defined three fertilizer efficiency parameters, including agronomic efficiency (AE), apparent nitrogen recovery (AR) and physiological efficiency (PE) (Eqns 4–6). AR reflects the efficiency of the crop in obtaining nitrogen-based fertilizer from the soil, whereas PE can be viewed as the efficiency with which crops use nitrogen in the plant for the synthesis of grain. These definitions differ from Moll *et al.* [12] in that the analysis uses an unfertilized control as the initial starting point for analysis. These equations (Eqns 3–8) can also be expanded to include additional factors, including physiological ones [12].

Table I. Definitions and formulae used to describe nutrient use efficiency in plants

Eqn	Term	Formula	Definition	Comments	Refs
1	Nitrogen use efficiency	$NUE = Sw \div N$	Sw, shoot weight (DW); N, nitrogen content of shoots (DW)	Does not account for biomass increases	[10]
2	Usage index	$UI = Sw \times (Sw \div N)$	Sw, shoot weight; N, nitrogen in shoots	Takes into account absolute biomass increase	[11]
3	Nitrogen use efficiency (grain)	$NUE = Gw \div Ns$	Gw, grain weight; Ns, nitrogen supply (g per plant)	Reflects increased yield per unit applied nitrogen	[12]
4	Uptake efficiency	$UpE = Nt \div Ns$	Nt, total nitrogen in plant; Ns, nitrogen supply (g per plant)	Measures efficiency of uptake of nitrogen into plant	[12]
5	Utilization efficiency	$UtE = Gw \div Nt$	Gw, grain weight; Nt, total nitrogen in plant	Fraction of nitrogen converted to grain	[12]
6	Agronomic efficiency	$AE = (Gw_F - Gw_C) \div N_F$	N_F, nitrogen fertilizer applied; Gw_F, grain weight with fertilizer; Gw_C, grain weight of unfertilized control	Measures the efficiency of converting applied nitrogen to grain yield	[7]
7	Apparent nitrogen recovery	$AR = (N_F \text{ uptake} - N_C \text{ uptake}) \div N_F \times 100$	N_F uptake = plant nitrogen (fertilizer); N_C uptake = plant nitrogen (no fertilizer); N_F = Nitrogen fertilizer applied	Measures the efficiency of capture of nitrogen from soil	[7]
8	Physiological efficiency	$PE = (Gw_F - Gw_C) \div (N_F \text{ uptake} - N_C \text{ uptake})$	Gw_F, grain weight (fertilizer); Gw_C, grain weight (no fertilizer)	Measures the efficiency of capture of plant nitrogen in grain yield	[7]

have been overexpressed using either constitutive or inducible promoters (Table 1).

It has been demonstrated that the overproduction of NR, when driven by the 35S promoter, reduced nitrate accumulation in leaves of *Nicotiana plumbaginifolia* [22]. These lower concentrations of nitrate were accompanied by higher foliar glutamine and malate levels. The use of the 35S promoter to drive NR gene expression allows for a deregulated transcription of the NR gene but the NR protein is still controlled post-translationally by a phosphorylation mechanism that inhibits the enzymatic activity of NR through binding of a 14-3-3 protein [23]. An NR protein lacking 56 amino acids from its N-terminal domain was expressed in *N. plumbaginifolia* and shown to lack the post-translational regulation by light [24]. However, the NR protein seems to be phosphorylated and bound to endogenous 14-3-3 proteins *in planta* [23,24]. Although Ferrario-Méry *et al.* [25] have been able to show an increase in NR activity in transgenic plants, they were unable to show any direct phenotype associated with this trait. Djennane *et al.* [26,27] introduced a deregulated NR gene under the control of the CaMV 35S promoter into potato. The transgenic plants did not show any increase in yield or tuber numbers, but they did show highly reduced nitrate levels in the tubers. Crété *et al.* [28] overexpressed nitrite reductase (NiR) in *Arabidopsis* and tobacco, and found that the transgenic plants did not show any phenotypic differences. Although NiR mRNA was strongly expressed in the transgenics, NiR activity and protein levels were significantly reduced in plants growing on medium containing ammonium, suggesting that post-transcriptional regulation is operating on NiR expression. Takahashi *et al.* [29] demonstrated that transgenic *Arabidopsis* containing the NiR gene had higher NiR activity and higher rates of assimilation of NO_2.

In summary, the overexpression of NR seems to reduce the level of nitrate in the tissue analysed. Overexpression of either the NR or the NiR gene in plants has been shown to increase mRNA levels, and often affects N uptake. However, the increased uptake of N does not seem to increase the yield or growth of the plants regardless of the nitrogen source available. This is believed to be due, in part, to the complex regulation of NR and the pathway as a whole.

Glutamine synthetase and glutamate synthase

Following the discovery of the major role of the enzyme couple glutamine synthetase (GS) and glutamate synthase (GOGAT) in ammonium assimilation in higher plants [30], several laboratories have focused on understanding the mechanisms controlling the regulation of this pathway [31]. In addition, the generation of mutants or transgenic plants with altered levels of GS/GOGAT have been used to determine the effects of these proteins

Table 1. Nitrogen use efficiency in transgenic plants expressing genes involved in nitrogen uptake and metabolism

Gene	Gene product (cellular role)	Source	Promoter	Target plant	Phenotype observed	Refs
Nitrogen transporters						
Nrt1.1	Nitrate transporter (high affinity)	*Arabidopsis*	CaMV 35S	*Arabidopsis*	Nitrate uptake	[16]
NRT2.1	Nitrate transporter (high affinity)	*Nicotiana plumbaginifolia*	CaMV 35S; *rolD*	*Nicotiana tabaccum*	Nitrate content	[17]
Nitrate reductase, nitrite reductase						
Nia2	Nitrate reductase	*Nicotiana tabaccum*	CaMV 35S	*Solanum tuberosum*	Reduced nitrate levels	[26,27]
Nia	Nitrate reductase	*Nicotiana tabaccum*	CaMV 35S	*Lactus sativa*	Nitrate content, chlorate sensitivity, nitrate levels	[67]
Nia	Nitrate reductase (Ser521 mutation)	*Nicotiana tabaccum*	CaMV 35S	*N. plumbaginifolia*	NR activity, nitrate accumulation	[68]
NR	Nitrate reductase	*N. plumbaginifolia*	CaMV 35S	*Nicotiana tabaccum*	NR activity, NR transcript	[69]
NR	Nitrate reductase	*N. plumbaginifolia*	CaMV 35S	*Nicotiana tabaccum*	Biomass, NR activity, drought stress	[70]
NiR	Nitrite reductase	*Spinacia oleracea*	CaMV 35S	*Arabidopsis*	NO_2 assimilation	[29]
NiR	Nitrite reductase	*Nicotiana tabaccum*	CaMV 35S	*N. tabaccum*, *Arabidopsis*	NiR activity	[28]
Aminotransferases and dehydrogenases						
AspAT	Aspartate aminotransferase (synthesis of aspartate)	*Panicum miliaceum*	CaMV 35S	*Nicotiana tabaccum*	Enzyme activity, PEPC activity	[45]
GdhA	Glutamate dehydrogenase	*E. coli*	CaMV 35S	*Nicotiana tabaccum*	Plant biomass, DW, yield in field	[44]
ASN1	Asparagine synthetase (synthesis of asparagine)	*Arabidopsis*	CaMV 35S	*Arabidopsis*	Seeds with enhanced N status, N limitation tolerance	[51]
ASN1/ΔglnAS1	Asparagine synthetase, mutated glutamine synthetase	*Pisum sativum*	CaMV 35S	*Nicotiana tabaccum*	Growth rate, amino acid analysis	[50]
AtGluR2	Glutamate receptor	*Arabidopsis*	CaMV 35S2	*Arabidopsis*	Reduced growth rate, calcium use	[53]

Abbreviations: CaMV 35S, cauliflower mosaic virus 35S promoter; DW, dry weight; NiR, nitrite reductase; NR, nitrate reductase; PEPC, phosphoenolpyruvate carboxylase; *rolD, Agrobacterium rhizogenes rolD* promoter.

on plant development and to study the expression of the different members of the GS multigene family [32] (Table 2). The two GS isoforms are located either in the cytosol (GS1) or in the plastids (GS2) and have specific functions in assimilating or recycling ammonium [33]. GOGAT catalyses the reductant-dependent conversion of glutamine and 2-oxaloglutarate to two molecules of glutamate and occurs as two distinct isoforms, one ferredoxin dependent (Fd-GOGAT) and the other NADH dependent (NADH-GOGAT). Fd-GOGAT is the predominant form and plays an important role in leaf photorespiratory ammonium assimilation [33]. By contrast, NADH-GOGAT is found primarily in non-photosynthetic tissue, where it is the major form of GOGAT and combines with GS1 to assimilate NH_4^+ produced by nitrogen-fixing bacteria [34].

Several attempts have been made to study the function of different members of the GS/GOGAT genes in plants (Table 2). Although several studies have demonstrated an increase in GS activity in transgenic plants, many have been unable to show any direct increase in activity or phenotype associated with this trait (Table 2). For example, Ortega *et al.* [35] showed that transgenic alfalfa plants transformed with GS under the control of a CaMV 35S promoter accumulate transcripts without a corresponding increase in the level of enzyme activity. These results indicate that post-transcriptional controls are regulating higher levels of GS expression. Although these examples show no response or unusual phenotypic effects of GS overexpression (Table 2) [36,37], other studies have shown significant increases in plant biomass upon incorporating a novel GS1 construct. For example, Oliveira *et al.* [38] overexpressed the GS1 gene under the control of a CaMV 35S promoter and demonstrated that the transgenic plants had increased fresh weight, dry weight and leaf protein directly correlated with the increased level of GS in leaves. In continuation of this work, Fei *et al.* [39] produced transgenic peas overexpressing the cytosolic GS1 gene and demonstrated that the transgenic lines had a two- to eightfold increase in GS activity in the roots. In one of the two transgenic lines, increased GS activity resulted in lower N content and biomass accumulation at the four N treatments used (0 mM, 0.1 mM, 1.0 mM or 10 mM NO_3^-), whereas, for second line, biomass and N accumulation showed a 30% increase at 0.1 mM NO_3^- but were not affected at

Table 2. Glutamine synthetase/glutamate synthase and regulatory genes involved in nitrogen uptake and metabolism

Gene	Gene product (cellular role)	Source	Promoter	Target plant	Phenotype observed	Refs
Glutamine synthetase/glutamate synthase (GS/GOGAT)						
GS1	Glutamine synthetase (cytosolic)	*Pisum sativum*	CaMV 35S	*Nicotiana tabaccum*	Enhanced growth, leaf protein, ammonia levels	[38]
GS1	Glutamine synthetase (cytosolic)	*Pinus sylvestris*	CaMV 35S	*Hybrid poplar (Populus tremula*× *Populus alba)*	Enhanced growth, chlorophyll and protein	[71,72]
GS1	Glutamine synthetase (cytosolic)	*Phaseolus vulgaris*	Rubisco small subunit	*Triticum aestivum*	Enhanced capacity to accumulate nitrogen	[41]
GS1	Glutamine synthetase (cytosolic)	*Medicago sativa*	CaMV 35S	*Nicotiana tabaccum*	Shoot and root weight, enhanced growth	[73]
GS1	Glutamine synthetase (cytosolic)	*Medicago sativa*	*Srglb3p*[a]	*Lotus japonicus*	Sterility of the plants	[37]
GS1	Glutamine synthetase (cytosolic)	*Glycine max*	*RolD*	*Lotus japonicus*	Decrease in biomass	[74]
GS1	Glutamine synthetase (cytosolic)	*Glycine max*	CaMV 35S	*Lotus corniculatus*	Accelerate senescence	[36]
GS1	Glutamine synthetase (cytosolic)	*Glycine max*	CaMV 35S	*Medicago sativa*	No increase in GS activity	[35]
GS2	Glutamine synthetase (plastidic)	*Oryza sativa*	CaMV 35S	*Oryza sativa*	Enhanced photo-respiration, salt tolerance	[75]
GS2	Glutamine synthetase (plastidic)	*Nicotiana tabaccum*	Rubisco small subunit	*Nicotiana tabaccum*	Enhanced growth rate	[76]
Fd-GOGAT	Ferredoxin-dependent glutamate synthase	*Nicotiana tabaccum*	CaMV 35S	*Nicotiana tabaccum*	Diurnal changes in ammonia assimilation	[77]
NADH-GOGAT	NADH-dependent glutamate synthase	*Oryza sativa*	*O. sativa* NADH-GOGAT[b]	*Oryza sativa*	Enhanced grain filling	[42]
NADH-GOGAT	NADH-dependent glutamate synthase	*Medicago sativa*	CaMV 35S	*Nicotiana tabaccum*	Higher total carbon and nitrogen content in shoots, dry weight	[78]
Regulatory and transcription factors						
ANR1	MADS transcription factor	*Arabidopsis*	CaMV 35S	*Arabidopsis*	Root length	[79]
Dof1	Transcription factor and activator associated with carbon metabolism	*Zea mays*	35SC4PDK[c]	*Arabidopsis*	Growth rate under nitrogen-limiting conditions	[54]
GLB1	PII regulatory protein	*Arabidopsis*	CaMV 35S2	*Arabidopsis*	Growth rate, anthocyanin production	[56]

[a] *Sesbania rostrata* leghemoglobin gene promoter.
[b] *Oryza sativa* NADH-dependent glutamate synthase promoter.
[c] CaMV 35S promoter with TATA box and the transcription site of the maize *C4PPDK* gene.

0 mM, 1.0 mM or 10 mM. These results suggest that overexpression of GS does not consistently result in increase in GS activity and that the increase in GS activity is not always consistent with decreases in plant N and biomass accumulation. Four studies have reported increased growth rates in transgenics over-expressing GS1 and, in all cases, this occurred under low N conditions (Table 2).

Several studies have demonstrated a direct correlation between an enhanced GS activity in transgenic plants and biomass or yield [38,40,41]. When transgenic wheat lines expressing the *Phaseolus vulgaris* GS1 gene under the control of rice Rubisco small subunit (*rbc*S) promoter were grown in pots to maturity and their productivity analysed, they demonstrated an enhanced capacity to accumulate nitrogen in the plant. In addition, one line showed significantly higher root and grain yield, and the grain had a higher N content [41]. All these studies suggest that the overall level of nitrogen assimilation can be enhanced using the GS1 genes.

In comparison to GS, few reports have described the production of transgenic plants overexpressing GOGAT genes. Transgenic plants overexpressing an alfalfa GOGAT gene showed an increase in GOGAT protein content but did not show any phenotype associated with this trait (Table 2). However, Yaa *et al.* [42] overexpressed NADH-GOGAT in rice under the control of its own promoter and found that transgenic rice plants showed an increase (up to 80%) in grain weight. This study showed that overexpression of NADH-GOGAT can be used as a key step for nitrogen use and grain filling in rice and other cereal crops. In summary, results with transgenic plants expressing transferred GS or GOGAT genes suggest that there could be ways in which it is possible to improve the efficiency with which crop use nitrogen. Further characterization is required to demonstrate the beneficial effect of overexpression of GS and

GOGAT genes in N assimilation, which will ultimately lead to improvements in agronomic crops.

Other gene systems regulating N metabolism

Although the GS/GOGAT enzymes are the primary routes of ammonium assimilation in plants, the physiological role of glutamate dehydrogenase (GDH) has been less clear [43]. Ameziane *et al.* [44] investigated the role of GDH by expressing a bacterial *gdhA* gene from *E. coli* in tobacco under the control of the CaMV 35S promoter. They found that biomass production was consistently increased in *gdhA* transgenics, regardless of whether they were grown under controlled conditions or in the field. Sentoku *et al.* [45] have analysed the overexpression of both cytoplasmic and mitochondrial aspartate aminotransferase (mAspAT) genes using the CaMV 35S promoter in tobacco. They did not report growth or biomass data, although they did observe [using an antibody against maize phosphoenolpyruvate carboxylase (PEPC)] that the overexpression of the mAspAT gene resulted in the induction of the endogenous PEPC gene. In higher plants, asparagine synthetase (AS) catalyses the formation of asparagine (Asn) and glutamate from glutamine (Gln) and aspartate, and is encoded by a small gene family (*ASN1*, *ASN2*, *ASN3*) [46]. Thus, together with GS, AS is believed to play a crucial role in primary nitrogen metabolism [30,47]. Because Asn is a long-distance nitrogen transport compound and has a higher N:C ratio than Gln, Asn can be used for long-range transport and storage compounds, which is vital to nitrogen assimilation and other physiological process [48]. The finding that the level of AS transcripts and polypeptides in the transgenic nodules of *Medicago truncatula* were constantly increased when GS was reduced suggests that AS can compensate for the reduced GS ammonium assimilatory activity [47]. However, the same authors have also demonstrated that GS activity is essential for maintaining the higher level of AS. Thus, GS is required to synthesize enough Gln to support Asp biosynthesis via NADH-GOGAT and aspartate aminotransferase (AAT) [47]. The reduction in GS activity in transgenic plants of *Lotus japonicus* was also found to be correlated with an increase in asparagine content [49]. Thus, this study further supports the notion that, when GS becomes limiting, AS be important in controlling the flux of nitrogen into plants. With the aim of increasing Asn production in plants and to study the role of AS, several authors have attempted to clone AS genes and to examine the corresponding gene expression in plants [47,50–52]. Brears *et al.* [50] overexpressed two separate forms of AS in tobacco. The first type was normal, wild-type AS and the other had a deletion of the glutamate binding domain of AS (glnΔAS). Although both sets of transgenics had increased levels of free asparagine, the only significant difference in growth was a reduction in biomass for one of the glnΔAS transgenic lines. Glutamate receptors were originally identified in mammalian systems but recent completion of the *Arabidopsis* sequencing project has led to the identification of these genes in plants. Kim *et al.* [53] overexpressed an *AtGlu2* gene in *Arabidopsis* and demonstrated that overexpression of this gene resulted in plants that displayed Ca^{2+} deficiency. Overexpression of the *ASN1* gene in *Arabidopsis* has been found to enhance soluble seed protein content, total protein content and higher fitness of young transgenic seedlings when grown on nitrogen-limiting medium [51]. Recently, Wong *et al.* [52] produced transgenic *Arabidopsis* lines overexpressing or underexpressing the *ASN2* gene under the control of CaMV 35S promoter. When they grew transgenic plants on medium containing 50-mM ammonium, *ASN2* overexpressers accumulated less endogenous ammonium than the wild-type plants. When plants were subjected to high light irradiance, ammonium levels increased. These studies demonstrate that it is possible to manipulate the nitrogen metabolism and growth phenotype of plants by overexpressing AS genes and that this might thus be one way to improve nitrogen use efficiency in crop plants.

Yanagisawa *et al.* [54] overexpressed in *Arabidopsis* the maize transcription factor Dof1. Maize Dof1 is a member of a transcription factor family unique to plants [54] and an activator for multiple genes associated with organic acid metabolism, including PEPC gene expression [54]. They found that the ectopic expression of Dof1 induced the upregulation of genes involved in carbon skeleton production and resulted in a marked increase in amino acid content in the transgenics. More significantly, the Dof1 transgenics exhibited improved growth under low-nitrogen conditions. Successful molecular approaches towards improved N use in plants also include the modification of proteins that coordinate the regulation of N and C metabolism. Two proteins from *Arabidopsis*, a bacterial PII-like protein and a putative glutamate receptor, have been linked to N and C sensing and regulation as observed in biochemical and transgenic plant studies [55,56]. The possible central role of these proteins in N and C metabolism indicates that they are good candidate proteins for engineering through the targeted modification of their sensory or regulatory domains. The observation that the putative glutamate receptor, when its expression is reduced in antisense lines, resulted in reduced expression of the cytosolic isoforms of GS and AAT demonstrates the importance of this regulatory protein on N metabolism.

Challenge of manipulating nitrogen remobilization

Much of the research on NUE has focused on nitrogen uptake from the soil and its metabolism and transport to the leaves. However, in cereals and other crops, grain yield is based not only on nitrate uptake before flowering but also on the remobilization of leaf N during seed maturation (Box 2). The ability to remobilize leaf nitrogen is also subject to genetic variability because, among the wheat cultivars analysed, the proportion of N accumulated by the spike from leaf nitrogen varied from 51% to 91% [57]. During the past few years, several laboratories have identified genes encoding proteins that are specifically activated during the remobilization of nitrogen, carbon and minerals during leaf senescence [58]. In addition, efforts are being made to study the biochemical mechanisms involved in N export and import from source and sink leaves during senescence [59,60]. Interestingly, an increase in the activity of GS and GDH was observed during leaf senescence [61]. Vincent *et al.* [36] studies the overexpression of GS1 in *Lotus corniculatus* and observed

a large increase in the amino acid content of roots and shoots, and premature flowering. These observations suggest that N remobilization was induced artificially by the overexpression of GS. In spite of the studies conducted over the past few years both at the whole plant level and using transgenic plants, understanding the mechanisms involved in N remobilization during leaf senescence and remobilization is still at a preliminary stage and requires more research.

Integration of genetic and molecular approaches

Early attempts to evaluate the genetic basis of NUE in plants were restricted to simple genetic models. However, with the development of molecular markers, evaluating the inheritance of NUE became a more tractable problem because specific quantitative trait loci (QTLs) could be identified. QTLs for NUE have now been identified in mapping populations of maize, rice, barley and *Arabidopsis* [62–65]. In maize, Hirel *et al.* [62] and Gallais and Hirel [58] have analysed recombinant breeding lines for NUE, having already assessed these lines for several physiological traits such as nitrate content and NR and GS activity. When the variation in physiological traits and yield components were compared, a positive correlation was observed between nitrate content, GS activity and yield. Loci that appeared to govern quantitative traits were identified on the maize genome map and the positions of the QTLs for yield components and the location of the genes for cytosolic GS were shown to coincide. Recently, Obara *et al.* [63] followed a similar line of research in rice and, again, coincidental locations were found for a QTL for a yield trait and a structural gene for GS1. These results suggest that GS1 might be a key component of nitrogen use efficiency and yield.

Although it is not our purpose in this article to cover the genetics of NUE (for this, see the review by Gallais and Hirel [58]), two points are worth noting. Even though map-based cloning is labour intensive, we hope that identifying candidate genes and analysing their expression patterns will allow us to focus more quickly on genes that improve NUE. Second, we believe that, for complex metabolic traits such as NUE, the use of transgenics will need to be more tightly integrated with classical breeding and marker-assisted selection if these introduced genes are to provide the maximum benefit. To date, when molecular biologists have introduced a gene into a plant, the level of expression of the gene has been known to display 'position effect' phenomenon. However, it is now being recognized in other systems that genetic background can have a significant effect on transgene expression [66]. We anticipate that the effectiveness of a specific transgene for traits such as NUE will be based on the specific genetic environment into which it is placed, independent of the position effect.

Summary

There are two key requirements to identifying and understanding the regulation of genes important in enhancing nitrogen use efficiency. First, there is a need for proper evaluation of NUE as a component of any crop improvement program aimed at increasing NUE. Many breeders might argue that, by selecting for yield, one is by definition selecting for nutrient efficiency. However, unless a breeder specifically evaluates a crop for NUE, the benefit of growing 'efficient' crops will not be recognized. Second, although there have been a few successes in manipulating N metabolism in plants such as tobacco or *Arabidopsis* using specific genes, these traits now need to be evaluated in economically important crop plants. Moreover, even though researchers tend to focus on a few basic plant systems, there has also been no attempt to observe the effectiveness of the transgene expression in different genetic backgrounds. Given that the global human population is expected to reach ten billion by the year 2070, feeding all these people will require more efficient use of agricultural lands [2,3]. We believe that creating crops with enhanced nutrient uptake will be one component that will help us to achieve this goal.

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