Genetic Engineering Approaches to Improving Nitrogen Use Efficiency

Ashok K. Shrawat and Allen G. Good

Since nitrogen (N) is the most essential nutrient for plants and a major limiting factor in plant productivity, doubling agricultural food production worldwide over the past four decades is associated with a 20-fold increase in N fertilizer use. As a consequence, use of N fertilizers in agriculture has already shown a number of detrimental environmental impacts. Therefore, the need to reduce N fertilizer pollution is strengthening the importance of improving the nitrogen use efficiency (NUE) of crop plants. The development of crop plants that take-up and assimilate N more efficiently would reduce the need for N fertilizers and positively influence the environment. Here, we discuss recent developments in the genetic manipulations of NUE in crop plants.

Background

Crop plants, especially grown for protein content and grain yield, require large quantities of inorganic N fertilizers[1]. Consequently, in the last 50 years the N fertilization of crop plants worldwide has increased more than 20-fold. However, use of this fertilizer is generally inefficient, as only about a third of the fertilizer applied is actually absorbed by crops, and 50 – 70% is lost from the plant-soil system[2]. Unused fertilizer can leach into the environment, where it induces algal blooms, contaminates drinking water, and depletes aquatic oxygen to create dead zones, like those found in the Gulf of Mexico. Recently, Johnson and colleagues[3] showed that elevated nutrient inputs into aquatic ecosystems due to heavy use of N and phosphorus leads to eutrophication and increases pathogenic infection in amphibians. Because of the heavy use of N fertilizers, which is one of the major costs associated with the production of high-yielding crops and is the source of environmental damage due to excess N that is not taken up by plants[4,5], there is significant interest in genetic engineering crops to improve NUE[6-8].

Engineering plants with transport gene systems

Crop plants obtain N from the soil primarily as nitrate or ammonia, although some plants utilize amino acids as significant sources of N. Following uptake by specific transporters located in the root cell membrane, nitrate is reduced to ammonium through the combined action of nitrate reductase (NR) and nitrite reductase (NiR). In higher plants, the expression of the NR genes is influenced by several external and endogenous factors and is highly regulated at the transcriptional as well as post-translational levels[9]. The overexpression of either the NR or the NiR gene in plants increases mRNA levels and often affects N uptake. However, the increased uptake of N does not seem to increase the yield or growth of plants, regardless of the N source[6,7]. This is believed to be due, in part, to the complex regulation of both NR and the pathway as a whole. Recently, Lea et al.[10] demonstrated that post-translational regulation of NR strongly affects the levels of free amino acids, ammonium, and nitrate, whereas transcriptional regulation has only minor influence. Plants expressing fully unregulated NR accumulate high concentrations of asparagine and glutamine in leaves; however these transgenic plants grow and developed normally, despite having an NR enzyme that is active during both light and dark periods.

Glutamine synthetase and glutamate synthase gene systems

In higher plants, glutamine synthetase (GS) is represented by two groups of proteins—the cytosolic and plastidic forms[11]. A large number of studies on various plant species including both monocots and dicots show that cytosolic GS (GS1) is encoded by a complex multigene family, whereas plastidic GS (GS2) is encoded by a single gene[1]. Glutamate synthase (GOGAT) occurs as two distinct isoforms—ferridoxin and NADH-dependent—both of which are located in the plastid.

Since the discovery of the role of GS/GOGAT in ammonium assimilation in higher plants[12], there has been great interest in understanding the mechanisms controlling the regulation of this pathway[13]. Mutants or transgenic plants with altered levels of GS/GOGAT are used to determine the effects of these proteins on plant development and to study the expression of the different members of the GS multigene family[14].

Although several studies demonstrate that an increase in GS activity in transgenic plants has no effect on the phenotype, other researchers show a direct correlation between an enhanced GS activity in transgenic plants and an increase in biomass or yield, upon incorporating a novel *gs1* construct[6,8,15]. For example, tobacco plants overexpressing the *gs1* gene demonstrate increased fresh weight, dry weight, and leaf protein that is directly correlated with an

increased level of GS in leaves[16]. Fei et al.[17] produced transgenic peas overexpressing the cytosolic *gs1* gene and demonstrated that these transgenic lines have a two- to eightfold increase in GS activity in roots. Transgenic pea plants overexpressing the *gs15* gene under the control of a root specific promoter also demonstrate an increased biomass and N content[18]. However, inconsistent growth effects in the transgenic plants are also observed. Recently, poplar trees transformed with a conifer *gs1a* gene demonstrate significant increases in leaf area, dry weight, and plant height, both in controlled environmental and field conditions. Interestingly, the differences are more striking at a low nitrate concentration. In addition, higher rates of ^{15}N incorporation into the transgenic plants further demonstrate that the transformed plants have increased NUE[19].

In comparison to GS, few reports have described the production of transgenic plants overexpressing *GOGAT* genes. The most interesting results were obtained by Yamaya et al.[20] who overexpressed *OsNADH-GOGAT1* in rice under the control of its own promoter and found that transgenic rice plants show an increase in spikelet weight (up to 80%). Plant heights and spikelet number are unaffected. This study shows that overexpression of *NADH-GOGAT1* can be used as a key step for N use and grain filling in rice and other cereal crops.

Engineering plants with other gene systems regulating N metabolism
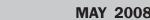
Over the past few years, attention was focused on the enzyme asparagine synthetase (AS), which catalyzes the formation of asparagine (Asn) and glutamate from glutamine (Gln) and aspartate. In higher plants, AS is encoded by a small gene family[21]. Together with GS, AS is believed to play a crucial role in primary N metabolism[13,22]. The observation that the levels of AS transcripts and polypeptides in the transgenic nodules of *Medicago truncatula* increase when GS is reduced suggests that AS can compensate for the reduced GS ammonium assimilatory activity[22]. However, the same authors 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 AspAT[22].

A reduction in GS activity in transgenic *Lotus japonicus* is also correlated with an increase in asparagine content[23], supporting the hypothesis that when GS becomes limiting, AS may be important in controlling the flux of reduced N into plants. With the aim of increasing Asn production in plants and to study the role of AS, several researchers attempted to clone AS genes and to examine the corresponding gene expression in plants. For example, Lam and colleagues[24] overexpressed the *ASN1* gene in Arabidopsis and demonstrated that the transgenic plants have enhanced soluble seed protein content, enhanced total protein content, and better growth on N-limiting medium. Arabidopsis plants overexpressing the *ASN2* gene accumulate less endogenous ammonium than wild-type plants when grown on medium containing 50-mM ammonium. When plants are subjected to high light irradiance, ammonium levels increase[25]. Transgenic Arabidopsis plants overexpressing the maize Dof1 transcription factor demonstrate not only better growth under N limiting conditions, but also enhanced N assimilation[26]. This study indicates that signaling processes may provide an attractive route for metabolic engineering. In comparison to GS/GOGAT enzymes, the physiological role of glutamate dehydrogenase (GDH) has been less clear[27]. In an attempt to investigate the role of GDH by expressing a bacterial *gdhA* gene from *E. coli* in tobacco, Ameziane et al.[28] found that biomass production is consistently increased in *gdhA* transgenics, regardless of whether they are grown under controlled conditions or in the field.

The challenge of manipulating N remobilization

Remobilization of N in plants is a very complex metabolic process and is of major importance for plant productivity because it recycles organic N to young developing leaves and storage organs[29]. Therefore, 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. In rice, approximately 80% of the total N in the panicle arises from remobilization through the phloem from senescing organs[30]. During the past few years, efforts have been made to identify genes encoding proteins that are specifically activated during the remobilization of N, carbon, and minerals during leaf senescence[31]. In addition, several laboratories are studying the biochemical mechanisms involved in N export and import from source and sink leaves during senescence[29,32].

Since cytosolic GS (GS1) is only induced during leaf senescence, it has therefore been suggested that this enzyme reassimilates ammonium released from protein hydrolysis[33]. Several studies using transgenic tobacco demonstrate that genetic manipulation influences plant phenotype and amino acid metabolism when N is limiting[34]. During N remobilization in cereals, GS1 facilitates the synthesis of Gln, which is the major form of reduced N in phloem sap, and NADH-GOGAT1 is important in developing sink organs for the remobilization of Gln in rice[7]. Thus, the synthesis of

Gln in senescing organs is considered a key step in N recycling.

A large increase in the amino acid content of roots (primarily) and shoots and premature flowering are observed in *Lotus corniculatus* overexpressing a soybean gene (*gs15*), which encodes cytosolic GS[35]. [15]N labeling experiments further demonstrate that both ammonium uptake in roots and the subsequent translocation of amino acids to shoots is lower in plants overexpressing *gs15*. These results suggest that the accretion of ammonium and amino acids in roots is due to shoot protein degradation. These results further confirm that N remobilization is induced artificially by the overexpression of *gs15*.

When transgenic wheat lines expressing the *Phaseolus vulgaris gs1* gene are grown in pots to maturity and their productivity analyzed, they demonstrate an enhanced capacity to accumulate N in the plant. Measurement of the total N content of tissue at harvest shows that transgenic plants with extra GS1 protein accumulate more N in their shoots and grain[36]. Although only one transgenic line showed improved N assimilation in one study, this indicates that genetic transformation of plants with GS may have a practical effect on NUE.

Recently, the roles of two genes encoding cytosolic maize GS1 (*gln1-3* and *gln1-4*) were investigated in detail by examining the impact of knockout mutations on kernel yield and by overexpressing *gln1-3* in maize[37]. The authors found that *gln1-4 gln1-3* double mutants display reduced kernel size and reduced kernel number, with no reduction in shoot biomass production at maturity. When maize is genetically transformed by constitutively overexpressing *gln1-3* using a cassava vein mosaic virus promoter, a significant increase in grain yield is observed (~30%). Again, there are no significant differences in shoot dry matter production between WT plants and the transgenic lines, which suggests the specific impact of g*ln1-3* on grain production. Transgenic maize plants overexpressing the *gln1-3* gene produce greater kernel numbers under both high and low N conditions when compared to wild type plants[15]. These studies on maize clearly suggest that GS1 plays an important role in kernel yield under high and low N fertilization. The reaction catalyzed by GS1, therefore, may be one of the key elements controlling crop yield.

In rice, GS1 knock-out mutants made by inserting the retrotransposon *Tos17* into exon-8 or exon-10 of *Osgs1;1* exhibit a severe reduction in growth and grain filling when grown using normal N fertilizer concentration. Reintroduction of the *Osgs1;1* cDNA under the control of its own promoter into the mutants successfully complements the slow growth phenotype. This study further indicates that GS1;1 is important for normal growth and grain filling in rice. GS1;2 and GS1;3 are not able to compensate for the function of GS1;1[38,30].

Summary

Studies with transgenic plants overexpressing genes affecting the N metabolism pathway suggest it is possible to improve or manipulate N metabolism and the growth phenotype of plants, which can improve the NUE of crop plants. However, in spite of 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.

In their excellent review article, Hirel and Lemaire[34] emphasize that for relatively long periods during vegetative growth, plant nutrition is near a steady state condition. However, after anthesis, crops experience a rapid exhaustion of the available N in soil and therefore grain filling has to be directly supported by N recycling. An improved understanding of the transition between N assimilation and N recycling will undoubtedly be of tremendous importance in applying transgenic approaches to improving the NUE of crop plants.

In order to further identify and understand the regulation of the genes involved in enhancing NUE, proper evaluation of the combined genetic and transgenic approaches to improving NUE should be required as a component of any crop improvement program. The benefits of growing NUE-efficient crops will not be realized until breeders evaluate N metabolism and nitrogen use efficiency in economically important crop plants. Given that the global human population is expected to reach ten billion by 2070, feeding everyone will require the more efficient use of agricultural lands, and creating crops with enhanced nutrient uptake will be one component in achieving this goal.

References
1. Lam H-M et al. (1996) *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **47**, 569-593
2. Peoples MB et al. (1995) In: *Nitrogen Fertilizer in the Environment*, Bacon PE, ed., pp. 565-606. Marcel Dekker
3. Johnson PTJ et al. (2007) *Proc. Natl. Acad. Sci. U.S.A*. **104**, 15781-15786
4. Vitousek P et al. (1997) *Ecol. Appl*. **7**, 737-750

5. Atkinson D et al. (2005) *Ann. Appl. Biol*. **146**, 203-215
6. Good AG et al. (2004) *Trends Plant Sci*. **9**, 597-605
7. Andrews M et al. (2004) *Ann. Appl. Biol*. **145**, 25-40
8. Lea PJ, Azevedo RA (2007) *Ann. Appl. Biol*. **151**, 269-275
9. Meyer C, Stitt M (2001) In: *Plant Nitrogen*, Morot-Gaudry JF & Lea PJ, eds., pp. 37-59. Springer
10. Lea US et al. (2006) *Plant Physiol*. **140**, 085-1094
11. Hirel B et al. (1993) In: *Control of Plant Gene Expression*, Verma, D.P.S, ed., pp. 443-458. Boca Raton, FL: CRC Press
12. Miflin RD, Lea PJ (1976) *Phytochem*. **15**, 873-885
13. Harrison J et al. (2000) *Plant Soil* **221**, 81-93
14. Coschigano KT et al. (1998) *Plant Cell* **10**, 741-752
15. Hirel B et al. (2007) *J. Exp. Bot*. **58**, 2396-2387
16. Oliveira IC et al. (2002) *Plant Physiol*. **129**, 1170-1180
17. Fei H et al. (2003) *Planta* **216**, 467-474
18. Fei H et al. (2006) *Plant Phyiol. Biochem*. **44**, 543-550
19. Man HM et al. (2005) *New Phytol*. **167**, 31-39
20. Yamaya T et al. (2002) *J. Exp. Bot*. **53**, 917-925
21. Lam H-M et al. (1998) *Plant J*. **6**: 345-353
22. Carvalho HG et al. (2003) *Plant Physiol*. **133**, 243-252
23. Harrison J et al. (2003) *Plant Physiol*. **133**, 253-262
24. Lam H-M et al. (2003) *Plant Physiol*. **132**, 926-935
25. Wong H-K et al. (2004) *Plant Physiol*. **134**, 332-338
26. Yanagisawa S et al. (2004) *Proc. Natl. Acad. Sci. U.S.A*. **101**, 7833-7838
27. Dubois F. et al. (2003) *Plant Physiol. Biochem*. **41**, 565-576
28. Ameziane R et al. (2000) *Plant Soil* **221**, 47-57
29. Masclaux C et al. (2001) *Ann. Appl. Biol*. **138**, 69-81
30. Tabuchi M et al. (2007) *J. Exp. Bot*. **58**, 2319-2327
31. Gallais A, Hirel B (2004) *J. Exp. Bot*. **55**, 295-306
32. Hayakawa T et al. (1994) *Planta* **193**, 455-460
33. Brugière N et al. (2000) *Planta* **211**, 519-527
34. Hirel B, Lemaire G (2005) *J. Crop Imp*. **15**, 213-257
35. Vincent R et al. (1997) *Planta* **201**, 424-433
36. Habash DZ et al. (2001) *Ann. Appl. Biol*. **138**, 83-89
37. Martin A et al. (2006) *Plant Cell* **18**, 3252-3274
38. Tabuchi M et al. (2005) *Plant J*. **42**, 641-651

Ashok K. Shrawat and Allen G. Good
Department of Biological Sciences, G-425, Biological Sciences Building
University of Alberta, Edmonton, Alberta, Canada, T6G 2E9
ashrawat@ualberta.ca