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The Genetics of Nitrogen Use Efficiency in Crop Plants

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Abstract

In the past 50 years, the application of synthetic nitrogen (N) fertilizer to farmland resulted in a dramatic increase in crop yields but with considerable negative impacts on the environment. New solutions are therefore needed to simultaneously increase yields while maintaining, or preferably decreasing, applied N to maximize the nitrogen use efficiency (NUE) of crops. In this review, we outline the definition of NUE, the selection and development of NUE crops, and the factors that interact with NUE. In particular, we emphasize the challenges of developing crop plants with enhanced NUE, using more classical genetic approaches based on utilizing existing allelic variation for NUE traits. The challenges of phenotyping, mapping quantitative trait loci (QTLs), and selecting candidate genes for NUE improvement are described. In addition, we highlight the importance of different factors that lead to changes in the NUE components of nitrogen uptake efficiency (NUPE) and nitrogen utilization efficiency (NUTE).

INTRODUCTION

Nitrogen use efficiency (NUE):

efficiency to uptake and utilize biologically reactive nitrogen from the growth medium, NUE = NUpE × NUtE = grain yield (Gw)/supplied N (Ns) The past five decades have seen some remarkable changes and advances in crop production, from the Green Revolution in the early 1960s to the advent of modern biotechnology in the 1990s. Improved crop management and agronomy combined with improved crop genetics through conventional breeding and genetic engineering have been the major factors behind increased crop production. Crop genetic improvement has been responsible for 50% to 60% of the increases in crop yields and is still a crucial component of any strategy to increase crop yields and nutrient use efficiency (41). Additionally, the large yield increases have also been due to the use of synthetic nitrogen (N), phosphorous (P), and potassium (K) fertilizers. One example of high fertilizer use and increased yields can be seen in the maize breeding programs, where breeders have selected for high seed-density-tolerant, high-yielding genotypes, in synchrony with high levels of applied N fertilizer (40, 42, 98).

Currently, there are two key concerns regarding crop yields and fertilizer application. The first is that traditional breeding strategies to improve crop plants may have reached a plateau. According to the Food and Agriculture Organization of the United Nations, since the mid-1980s global cereal crop yields, including wheat, soy, and maize, have slowed to a growth rate of about 1% annually, and in developed countries, growth of crop yields is closer to zero (47). Thus, it is clear that we face challenges in increasing yield, and many yield predictions are unrealistic (108). The second concern is that further increases in applied N may not result in yield improvements but will lead to serious environmental problems (27, 34, 56, 88, 130, 134, 141). Although the application rates of synthetic N fertilizers used in the United States on most crops increased following World War II, they accelerated markedly after the mid-1960s but have leveled off since the 1980s (32, 59). In contrast, in China between 1980 and 2010, the rate of cereal grain yield increased by 65%; however, the consumption of chemical fertilizers increased by 512% (29, 139). As a consequence, total crop yields in many intensive farming systems have failed to increase in proportion to the increase of chemical fertilizers over the past 20 years, leading to lownutrient use efficiency and increasing environmental problems (109). To achieve further increases in yield under well-fertilized conditions, we need to select for plants that use fertilizers more efficiently. Simultaneously achieving high crop productivity and high nutrient use efficiency is a major challenge, given the increased global demand for food, feedstock, and biofuels (26, 27, 55, 117). New solutions are needed to increase yields while maintaining, or preferably decreasing, applied N (56, 62).

In this review, we discuss the challenges of developing crop plants with enhanced nitrogen use efficiency (NUE), using more classical genetic approaches based on utilizing existing allelic variation for NUE traits. The alternative of using a transgenic approach is not discussed, as the key aspects of this have been covered in a number of recent reviews (10, 81).

THE BENEFITS OF IMPROVING NITROGEN USE EFFICIENCY

There are two main incentives to improve NUE in crop plants in order to use less N fertilizer: one is the economic gain, the other is N pollution reduction. Although the economic costs associated with inefficient N use are difficult to assess, Raun & Johnson (103) have estimated that every 1% increase in N fertilizer uptake efficiency would result in an approximate US \$2.3 billion savings in annual N fertilizer costs. In Canada, N fertilizers represented approximately 10% (or US \$3.4 billion) of the total farm operating costs in 2010. With annual growth rates of approximately 5%, it is estimated that increased annual fertilizer purchases will add approximately C \$170 million in costs per year to Canadian farmers (3). In addition, there are the unpredictable variations in

costs due to fluctuations in the price of the fossil fuels used to synthesize N fertilizers. Although challenging for all farmers, this volatility has been linked to recent increases in commodity prices and the resulting political instability in many Arab nations over the past decade.

There are many concerns about the negative impact of excess N on the environment. In Europe, the societal damage costs of excess N (e.g., water and air quality) have been estimated to be US \$91–\$466 billion annually (115). These numbers are significantly greater than the costs of excess N estimated by Beatty & Good (10), although it is difficult to calculate these costs with any degree of precision.

DEFINING NITROGEN USE EFFICIENCY

The fundamental approach to developing cultivars with enhanced NUE, in contrast to just improved yield, requires evaluating the plants under both low and high N conditions so as to identify a NUE-efficient genotype and compare its performance with that of other genotypes under various N conditions. Alternatively, one can measure N content directly in different tissues or developmental stages to calculate NUE. A number of different definitions and calculations of NUE include N utilization, N content, and N availability as NUE equation components (38, 49, 57, 85, 86). NUE comprises two key components: N uptake efficiency (NUpE), which is the efficiency of absorption/uptake of supplied N, and N utilization efficiency (NUtE), which is the efficiency of assimilation and remobilization of plant N to ultimately produce grain (57). The simplest definition of plant NUE is the grain yield per unit of supplied N, also represented by the product of NUpE and NUtE. Another method to describe NUE is the utilization index (UI), which factors in the absolute amount of biomass produced per unit of N. NUE can also be described as NUEg, which is grain production per unit of N available. However, a crop plant could produce large amounts of biomass per unit N (high UI) without converting the acquired N to seed production and therefore have a low NUEg. Other NUE calculations take various agronomic and physiological variations into account and are described elsewhere (30, 38, 49, 57, 62). In theory, improving NUE could be achieved by improving either NUpE, NUtE or both.

It is only in the recent past that plant breeders and geneticists have begun to select and analyze for improvements in NUE directly. Breeding and selection of improved varieties are generally done under high N supply, where the level of N is not limiting yield. This means that plant breeders select cultivars that perform well under these conditions, and therefore, by definition, if the selection criteria were for increased yield, then improved crop NUE was also selected for by default. The classical example of selecting for a plant's ability to utilize N efficiently was Norman Borlaug's introduction of dwarfing genes and selection for the resulting semidwarf high yielders. These genes (*Rht-B1* and *Rht-D1*), which were originally derived from a cross between a Japanese variety of dwarf wheat (Norin 10) and a high-vielding American variety (Brevor), became the model for the use of dwarfing genes to allow plants to use higher levels of N without the lodging common in the tall varieties (104). The dwarfing genes altered stem strength and plant architecture and indirectly generated plants that could produce much higher yield under high (standardized) levels of fertilizer and hence had enhanced NUE (104). However, since the selection of these highyielding, semidwarf wheat varieties on high N, what has been observed on a global scale, especially in developed countries, is a plateauing of any more yield increases, even while the crops are grown using the same high N levels. As NUE is a ratio of yield to N supply, this indicates that modern varieties are not improving in their NUE. Conversely, in many parts of the world, especially in developing countries, low-nutrient soils are the norm and often there are neither the finances nor the infrastructure to provide N-based fertilizers to the smallholder farmers. Geneticists now need to select genotypes/hybrids for their ability to yield under low N if they are developing these for

N uptake efficiency (NUpE):

the efficiency of absorption/uptake of supplied/available N, NUpE = plant N (Nt)/Ns

N utilization, or usage, efficiency

(NUtE): the efficiency of assimilation and remobilization of plant N to produce grain, NUtE = Gw/Nt

Utilization index

(UI): total plant biomass produced multiplied by the ratio of the total plant biomass to the total plant N

NUEg:

grain production per available N smallholder farmers. If there is a strong correlation between yields under high and low N, then a breeder can select for broad adaptability on nutrient-replete soils.

IMPROVING NITROGEN USE EFFICIENCY AND THE AVAILABILITY OF GENETIC VARIABILITY

Given the environmental heterogeneity that exists for nutrients in the soil, it is perhaps not surprising that there is a significant amount of genetic variation and phenotypic plasticity for NUE (20, 35). Therefore, in addressing whether we can use traditional genetics to improve NUE, one needs to determine the level of genetic variation present in the different landraces and genotypes of a crop. In order to parse out the contribution of N level from genetic and other environmental effects to plant yield, researchers need to be able to study a defined genetic population under different N conditions. However, as mentioned above, there are other factors to consider besides the genetics, such as the interactions between N uptake and water availability and the interaction between different macronutrients and micronutrients. When selecting for NUE, any variation in the environmental conditions could be as significant as genotype. If genotype rather than phenotype is used for selection, then an understanding of the genes that control the desired traits is also required. A large genotype by environment ($G \times E$) interaction on the expression of target traits is undesirable because it implies that the sought after improvements are not robust and may be observed only under specifically controlled conditions. In addition, the trade-offs associated with other desirable characteristics must also be considered. For example, modifying the root system to increase the uptake of nitrate from the subsoil could have negative effects on the uptake efficiency of less mobile, topsoil-located ions such as phosphate (14, 16, 68). In order to reconcile some of these conflicting demands and to help direct plant breeding, greater emphasis is currently being placed on the design of crop ideotypes for particular cropping systems and end uses.

FACTORS AFFECTING NITROGEN USE EFFICIENCY

The question of whether improvements in NUE result from NUpE, NUtE, or both has been looked at in maize, wheat, and barley. For maize, the relative contributions of NUpE and NUtE to overall NUE varies depending on a number of variables. Bertin & Gallais (13) found that in maize, NUpE was more closely correlated to NUE at high N and to NUtE at low N. Moll et al. (85) showed that maize hybrids with similar NUE differed considerably in uptake and utilization efficiencies, such that at low applied N, NUtE contributed more to the variation in NUE, whereas at high applied N, variation in NUE was almost entirely due to differences in NUpE. In contrast, Kamprath et al. (71) reported that genetic improvement of NUE in two maize populations was associated with increased NUpE at low N and increased NUtE at high N. In a comparison of hybrids derived from selection at low N versus high N, Presterl et al. (101) concluded that low-N-developed hybrids had superior N uptake compared with high N hybrids, and both groups had similar N utilization. NUE of tropical hybrids was influenced to similar extents by both NUpE and NUtE (132). Finally, the Illinois long-term maize experiments, which have run for more than 100 years, have demonstrated that one can perform divergent selection for increased grain protein concentration (GPC), and that this primarily impacts N utilization (125).

For wheat, the question of whether genetic gains in NUE can be explained by NUPE or NUtE depends on the level of N supply and on the study. Studies in the United Kingdom, Mexico, and Finland (50, 89, 97) all found that genetic gains in NUE were explained roughly equally by NUPE and NUtE, but that the importance depended on the level of available N. In an investigation of

wheat cultivars in France, NUpE accounted for more of the genetic variation in NUE at low N than at high N (76). In contrast, for 12 spring wheat varieties in California, NUpE was the most important component of NUE at both low and high N supply (37). In an examination of 39 cultivars at 5N rates in the United Kingdom, cultivar differences in N uptake were found only at the highest 3N rates, and NUtE explained more of the variation in grain yield than NUpE at all 5N rates (8). Similar observations were made by Ortiz-Monasterio et al. (97) and Le Gouis et al. (76), who observed that NUpE was more closely correlated to NUE at low N than at high N. This has also been found to be the case in other species, including barley (5) and oilseed rape (70).

Collectively, these studies suggest that the importance of NUpE and NUtE to NUE varies considerably depending on how these traits are measured or derived, the level of N fertilizer applied, and the germplasm under evaluation. Clearly, significant improvements in NUE require coordinated improvements of both NUpE and NUtE, and it is important to understand the interactions between uptake and utilization, as well as their genetic and environmental regulation (101).

THE CHALLENGES OF PHENOTYPING NITROGEN USE EFFICIENCY

One of the challenges to improving a phenotype (trait) determined by multiple genes is the collection of high-quality phenotypic data (90). For a complex trait like NUE, it is clear that although we have copious amounts of genetic and sequence data for each crop species, using that data meaningfully to improve phenotype is challenging. As such, experimenters should now focus on the implementation of appropriate experimental design that maximizes phenotyping efforts, rather than on the collection of more genotypic data. The selection of germplasm of appropriate levels of relatedness and the generation of high-quality phenotype data will be the major determinants of the ability to utilize the combined genetic and phenotypic data in the future (90). For example, it is difficult to compare genotypes selected in very different environments, owing to the distinctly different phenology of the genotypes. Many geneticists and breeders regularly evaluate germplasm from geographically diverse breeding programs; however, the North American, Australian, and European germplasm are all so distinctly different that making comparisons of NUE becomes problematic, owing to the many complicating factors affecting yield. A further complication is that for the development of commercial varieties of most crop plants, breeders stay within very limited germplasm (91), and therefore using quantitative trait locus (QTL) data from wide crosses that have generated relatively robust QTL information may not be relevant in developing modern crop varieties of maize, wheat, rice, and other cereal crops. Therefore, despite the significant investments in genomics, improving NUE, or for that matter any quantitative trait, will increasingly depend on the agronomist and accurate phenotyping than on genotyping.

Economically important traits are frequently polygenic; therefore, it is unlikely that a single genotype or recombinant inbred line (RIL) would contain the necessary contrast in the relevant genes underpinning the trait(s). For example, a trait controlled by 10 independent genes would require assessment of more than 3,000 backcrossed F_1 individuals to be 95% confident of identifying at least one F_1 individual heterozygous for all 10 alleles (17). Population sizes required for investigating polygenic traits are very large (>200 RILs) and therefore are resource intensive to phenotype confidently. If this is extended to multiple environmental backgrounds, different fertilizer treatments, and combined traits (e.g., higher yield while maintaining protein content), then the resources required become significant, even given the best genetic material and experimental design.

There is also the difficulty of measuring the trait reliably. Three to five percent increases in yield are fairly standard for differences in maize genotypes (112). However, in order to see this difference in the field, multiple field trials with large numbers of plots must be performed, which becomes very expensive if a large number of different genotypes are tested. Moreover, the researcher must

Quantitative trait locus (QTL): a statistical method that links phenotype (trait) to genotype (section of chromosome containing genes)



Figure 1

Nitrogen (N) response curve of three different genotypes differing in genetic N efficiency (yield at low N) and N responsiveness (ability to use synthetic N).

be aware of the many sources of inherent experimental variability, such as seed source, that must be accounted for when comparing different genetic lines to detect small quantitative changes in complex traits like NUE.

Several other issues must also be considered when analyzing NUE. First, as demonstrated in **Figure 1**, different genotypes may have very different abilities to use N fertilizers, based on the amount of applied N, which is determined by whether a producer applies significant amounts of synthetic N (developed world farmers) or not (developing world farmers). Genotype 1 is more efficient at low N and is more useful to a subsistence farmer in Africa; therefore, we say it has a high genetic N efficiency, whereas Genotype 2, which is the type that a breeder in the developed world would select, has a lower genetic efficiency but a higher N responsiveness. The ideal genotype has both high genetic N efficiency and high N responsiveness. Provided the traits for N efficiency and N responsiveness are not genetically linked (although they probably are to some degree), one should be able to select for genotypes that perform better under both conditions, requiring the evaluation of genotypes under different N conditions.

Second, one of the key concepts defining the relationship between the N that crops need and the N that harms the environment is nutrient synchrony (22, 31, 107). When N supply (via fertilizer application or organic matter mineralization) is in excess of crop N demand, they are asynchronous and N can accumulate in soils and be susceptible to various loss pathways (**Figure 2**) (58, 99). Current agronomic practices tend to set up the scenario where nutrient supply exceeds plant requirements early in the growing season, because N is applied when plant demand is low or nonexistent (e.g., in spring temperate annual cropping systems). Thus, the challenge becomes having a plant maximize its early N uptake; therefore, traits such as early vigor, an extensive root system, and efficient, developmentally early N-uptake systems are valuable in crops. Later in development, the ability to assimilate and remobilize the available N and associated carbon to the grain is also obviously crucial. **Figure 2** illustrates both this asynchrony and an additional problem associated with measuring NUE, that of developmental time by showing two

Genetic N efficiency: ability to produce high grain yield with no to low additional N fertilizer application

N responsiveness:

ability to respond to additional N applications with increased yield



Figure 2

Plant growth and nitrogen availability during plant development illustrating asynchrony and the importance of measuring nitrogen uptake efficiency and nitrogen utilization efficiency.

different genotypes, NUpE and NUtE, which differ in their growth rates, and the amount of available N in the soil. Different genotypes can show the same NUE and yield, even when their NUpE and NUtE are different, depending on the N accumulation in the plant at different times (either total biomass N or seed N). Genotype A is a slower growing plant that achieves lower total N content (lower NUpE) than Genotype B by the beginning of seed set; however, it has a higher NUtE due to a higher ratio between yield and total plant N. These two genotypes in the end produce the same N yield but in very different manners. For example, if Genotype B had 20% greater total N but was 20% less efficient at remobilizing the stored N into the grain, then these two genotypes would have the same N yield. However, if remobilization efficiency could be separated genetically from N storage, yield could be improved by 20%. **Figures 1** and **2** illustrate that geneticists and breeders need to consider whether they are interested in N genetic efficiency, N responsiveness, or both (9). It should be possible to develop genotypes that provide higher yield under lower N while maintaining the ability to convert applied fertilizer into yield.

TRAITS AFFECTING NITROGEN UPTAKE EFFICIENCY

Physiological traits that may affect NUpE include root architecture and any other characteristic that impacts the roots' ability to extract available N from the soil (49). A simple example of improving NUpE would be to increase uptake with expression of more efficient transporters, or increase expression of all the transporters; however, it is unlikely that upregulating transporters only would increase N uptake (61). A second example is to target genes identified to play a part in root architecture (54, 140). However, there have yet to be any examples in which the manipulation of these genes affects N uptake in a crop plant (81).

Studying NUpE and root systems, especially in soil, is still difficult and so it is one area that remains relatively unexplored. The capacity of the root for uptake depends on the degree to which the root extends its absorption area, which is determined by complex root morphology. The lateral roots, which occupy more than 90% of the total length of the root system, play a major role in water uptake. Despite their importance, it has proven difficult to perform field-based root phenotyping (61). To date, studies have been carried out to identify root morphological features

Nutrient synchrony: the extent to which the rate of crop N supply matched the rate of crop N demand N harvesting index (NHI): percentage of the total N allocated to the grain such as root mass and depth, root axis length, and lateral branching related to NUE (69, 110, 136). Although the development of better field phenotyping using various scanners looks promising, these techniques currently work only in controlled artificial conditions, and more labor intensive techniques, including field sampling of DNA to quantify the amount to root tissue, are both expensive and challenging.

TRAITS AFFECTING NITROGEN UTILIZATION EFFICIENCY

A number of physiological traits can affect the NUtE in crops, including the effect of N on carbohydrate partitioning, the storage of N, and the remobilization of N from senescent tissues, and these have been subdivided into a number of components by researchers (67, 77, 93). Gallais & Hirel (52) looked at several components of N remobilization, including stem remobilization, leaf blade remobilization, and total remobilization in a set of RILs in maize and identified many QTLs for these traits. Other researchers have examined N harvesting index (NHI). The minimum N% in the grain at harvest largely determines how much grain can be produced per unit of absorbed N. Van Keulen (128) concluded that the minimum level to which N can be diluted in small grains is approximately 1.6%, and Foulkes et al. (49) reported that current UK winter wheat cultivars had a minimum N% value of approximately 1.25%. If the NHI remains constant, then increasing NUtE results in decreased grain N content (GNC). Raising NUtE associated with lower grain N is feasible in end-use markets for which a high grain starch-to-protein ratio is desirable, e.g., the feed, distilling, and biofuel markets. A lower GNC implies more photosynthesis per unit of absorbed N, and there are a number of metabolic and physiological mechanisms by which this may potentially be achieved. These have been discussed in more detail by Foulkes et al. (49) and include a higher level of photosynthesis per unit N, better post-anthesis N remobilization, or the stay-green trait, which is the capacity of a genotype to retain a photosynthetic green leaf for longer than a standard genotype during grain filling (116).

DROUGHT: THE KEY FACTOR THAT INTERACTS WITH NITROGEN UPTAKE EFFICIENCY

Any analysis of different plant genotypes on yield, total N uptake, or NUE will demonstrate a genetic component to NUE, along with a strong environmental component and $G \times E$ (gene \times environment) interaction. However, are there environmental factors that can be monitored and used to provide a better understanding of the analysis required to enhance NUE? Clearly, the most important environmental factor affecting NUE efficiency is water availability. Without sufficient water, plants cannot extract nutrients from the soil and yield is constrained by moisture availability, not N availability. In maize, there are many examples in which drought and NUE have been shown to interact (reviewed in 7). Much of the effort has been to breed for varieties that grow well under the low N soil conditions, common in sub-Saharan Africa. Bänziger et al. (7) found that by selecting for drought tolerance, they were also able to increase the yield, regardless of the amount of additional N applied. Clearly, maize breeders need to consider N application rates when selecting for a drought-tolerant or water-efficient germplasm.

There are many examples from Australia and throughout the world of wheat grown under conditions in which moisture is periodically limiting. In these cases, because grain yield and N removal is driven by water supply, the uncertainties in rainfall will make for uncertainties in N application. In Australia, irrigated wheat yields were approximately 7 Mt/ha and required 125 kg/ha of fertilizer N, whereas dryland wheat yields were approximately 2 Mt/ha and required 27–40 kg/ha of fertilizer N (101a). Carberry et al. (24) reported that Australian rain-fed wheat

producers used only a tenth of the amount of fertilizer compared with Chinese irrigated wheat producers (27 kg/ha versus 260 kg/ha), but also averaged only 2.3 Mt/ha yield versus 6.5 Mt/ha.

Water availability affects NUE of barley as well. Analyzing 10 years of Canadian field trial data for barley showed that the year-to-year rankings of different cultivars for yield and NUE were consistent, except in those years where there was a drought and significant water stress on the plants (Y. Anbessa & A.G. Good, unpublished data). In canola, the negative effects of drought are quite similar to those for N limitation, with loss of grain yield, decline of seed oil content, and change in lipid composition (33, 92).

NITROGEN USE EFFICIENCY AND CULTIVAR DEVELOPMENT: HISTORICAL TO CURRENT

It should be recognized that for the majority of plants, their natural selection has primarily been to maximize the conservation of N within the plant tissues rather than the biomass per unit N (35, 113). Therefore, NUE in terms of evolutionary fitness is very different from NUE in an agricultural setting. Historically, the Long Term Broadbalk experimental plots implemented in 1843 at Rothamsted in the United Kingdom have provided a wealth of data that have documented the long-term changes in crop NUE (61). These historical plots have demonstrated the importance of N-based fertilizers, the increase in yield gains from improved crop genetics, and the effect of increased N fertilization levels on N loss into the environment (61). Many organizations, particularly the research stations associated with the CGIAR (Consultative Group for International Agricultural Research) Consortium, such as CIMMYT, have documented genetic variability for NUE in crop plants over multiple environments and years. By comparing the field performances of 10 varieties released from CIMMYT between 1950 and 1985, Ortiz-Monasterio et al. (97) showed that genetic gain for grain yield increase was associated with the improvement of NUE, indicating that breeders selected varieties with improved NUE unconsciously as a result of choosing higher-yielding varieties.

Maize

Many groups have evaluated different germplasm/hybrids for NUE, but only recently have varieties or hybrids been developed with this as a target. Maize is one of the best-studied crops, with many studies looking at yield increases and genetic gains in NUE, based on experiments that examined historic sets of hybrids and genotypes under both low and high N supplies. From the early 1960s to 1990, increased N fertilizer application accompanied early gains in grain yield of single-cross maize hybrids (43). However, with the average N fertilizer rates in the United States remaining relatively static since the early 1980s (126), the selection under a high N input has clearly resulted in maize breeders selecting for N-responsive germplasm (25).

Maize studies can be divided into two groups, namely comparison studies of hybrids from different eras and studies that use inbred lines or mapping populations to genetically dissect traits. Comparing hybrids from different historical times is a common approach to understanding how genetic selection has shaped economically important traits such as yield in maize (23, 28, 42, 131). However, the reality is that the impact of selection on N use has been well documented in only a few cases. Moreover, because most of the current proprietary maize germplasm in the United States used to generate hybrids can be traced to seven progenitor lines (83, 84, 91), the lack of genetic diversity might suggest that maize hybrids would not differ in their N response. However, early studies demonstrated that hybrids varied considerably in the N fertilizer rate required to maximize grain yield (114, 120–122). Several comparisons of older and newer hybrids under contrasting N levels have been reported (28, 45, 96, 118), the results of which showed

CGIAR (Consultative Group for International Agricultural Research) Consortium: global partnership uniting organizations like CIMMYT (international maize and wheat improvement center) in research for sustainable agricultural development that the newer hybrids gave greater yields at all levels of fertilizer application (25, 39). Recently CIMMYT breeders reported that, at least in the initial testing, they have developed maize hybrids that yield better on nutrient-depleted African soils and do virtually as well on nutrient-replete conditions (28a). The yield advantage of newer hybrids (compared with older ones) at lower levels of N fertilizer application indicates that NUE has improved over the decades. Although these comparisons seem relatively robust, many studies have shown that significant hybrid $\times N$ interactions exist, even in elite maize germplasm, that are specific to certain environments but are not of sufficient magnitude to merit hybrid specific N recommendations (19, 53, 87, 111). The differences between these studies suggest that the evaluation environment, the hybrids used in a particular study, or other agronomic parameters such as plant density can markedly influence the conclusions drawn from a hybrid \times N rate experiment. For example, Tsai et al. (120) suggested that environmental conditions that are not supportive of high grain yields or elevated residual soil N might obscure genetic differences in response to fertilizer N. Also, large differences in hybrid maturity and lack of adaptation to a particular environment have been proposed as explanations for the apparent genetic differences for N response (53). Thus, whether maize hybrids respond differently than inbred lines to fertilizer N remains difficult to determine. This variability and the limited range of multiple-year genetic studies with large data sets make any comparison unreliable.

Wheat and Barley

In bread wheat, a number of studies have looked at the genetic variation for components of NUE. Barraclough et al. (8) performed the most detailed study, looking at 39 different winter wheat varieties that varied by trait (e.g., height), heritage, and year of registry. Their study showed a large range in variability (between 24% and 42%) for NUE and grain yield, depending on N application rate. As their study represented elite lines, there is no question that there would be significant variation for NUtE in diverse wheat germplasm.

In an analysis of 75 years of breeding for increased grain yield in barley, Bingham et al. (15) found that breeding had resulted in 1.0% and 1.2% per year increases in yield and NUE, respectively. Overall, this increase resulted from the newer varieties having larger grains, a lower GNC, and more grains per unit area than the older varieties. Similarly, modern Argentinian varieties had greater NUE and NIH than older varieties (2). However, as with other small grain cereals, both these studies found that the increase of yield potential often reduced the GNC (21, 97). Thus, that which can be positive for grain yield may not be good for grain quality. In barley, the importance of GNC is widely known. Although percent protein is only one of several traits related to malting quality, ranging between 8.5% and 12.5%, this is the most important requirement of the malting industry (51). Because yield improvements in barley are associated with decreases in GNC (1), it is still well recognized that adding additional N fertilizer at the appropriate time is often required to produce high-quality malting barley.

MAPPING NITROGEN USE EFFICIENCY QUANTITATIVE TRAIT LOCI

The number of QTLs detected in a given study depends on a number of different factors, including the size and type of mapping population used, the trait being investigated, the number of environments used for phenotyping, environmental conditions, and genome coverage. QTLs reported in the literature generally fall into two groups of genes. The first group includes major genes, with very large effects on highly heritable traits, with each QTL explaining a large portion of the total trait variation in a mapping population. The second and largest group is regulated by many genes, each explaining a small portion of the total trait variation, and numerous QTLs of small additive effects are shared among families. Clearly, the genetic variation for many

quantitative traits involves a number of loci with moderate effects, and a very large number of loci with minor effects (72, 106). Major gene effects can be studied via segregation analysis, as well as evolutionary history; however, when numerous genes with minor effects determine a trait, it is much more challenging, as they usually cannot be investigated individually.

Despite this, researchers have mapped NUE traits in a number of plants. One of the first was carried out by Obara et al. (94), who studied rice. They looked at QTLs associated with NUE and determined whether they cosegregated with glutamine synthetase 1 (GS1) and NADH-GOGAT. The analysis identified seven loci that cosegregated with GS1 activity and six loci that cosegregated with NADH-GOGAT activity. A number of QTLs for agronomic traits related to N use and yield have been mapped to the chromosomal regions containing GS2 in wheat and rice (16, 48, 60, 75, 94, 100, 133, 137, 138, 142), suggesting that the genomic region surrounding GS2 may be valuable for breeding rice and wheat varieties with improved agronomic performance and NUE. However, to date, no one has been able to introduce a GS gene into a NUE-inefficient background and show either enhanced NUE or yield.

For maize, a number of studies have looked at QTLs associated with NUE using segregating maize populations (52). Liu et al. (79) did a meta-analysis for QTLs for yield and yield components identifying a number of meta-QTLs under low and normal N conditions and characterized these by map position, confidence interval, and the proportion of phenotypic variance explained. Meta-analysis revealed 22 QTLs under low N conditions with 4 consensus QTLs found on chromosomes 1 and 4, 2 on chromosomes 3, 5, 6, and 9, and 3 on chromosome 2. Chromosomes 7, 9, and 10 contained only 1 consensus QTL (79). However, these analyses were looking at yield under low N, which is not what some researchers would consider to be NUE. Regardless of the definition of NUE, these studies point out many of the challenges, as there are always multiple QTLs for the trait, and each QTL rarely accounts for a significant amount of variation.

Bordes et al. (18) identified 54 genomic regions associated with grain yield, its components, and other traits through whole-genome association mapping with 196 wheat accessions. The analysis also revealed that 23 genomic regions were N responsive, which may be useful for the wheat breeding programs aiming to improve N responsiveness. More recently, Xu et al. (135) detected QTLs from a wheat RIL population, applying a conditional analysis to detect N and P fertilization effects on QTLs. N treatment influenced QTLs of N concentration in grain, shoots, and NUtE traits, whereas P treatment affected a QTL of spikelet number per spike. It may be worthwhile to further investigate the newly detected QTLs, including those on chromosomes 4B and 7A for yield component traits (135). Some QTL studies were incorporated with physiological traits such as N metabolic enzyme activities and N metabolites (48, 60). Although those traits are particularly susceptible to environmental factors and experimental settings (e.g., field versus glasshouse), there are potential outcomes such as metabolic QTLs (mQTLs) that provide us direct evidence to link agronomic traits with potential gene(s) underlying the QTLs (65, 105).

Although there have been a number of QTL maps of barley, the only trait that is usually mapped is yield, and many QTLs, most with minor effects, have been shown to be associated with barley yield (6, 63). Varshney et al. (129) used an association mapping approach to look at genes involved in a number of traits in barley, with the traits grain yield and biomass yield considered to be associated with NUE. Using two separate sites, they were able to map several QTLs involved in these traits. Berger et al. (12) mapped markers associated with a number of key agronomic traits associated with NUE in a genome-wide association study of 329 genotypes of winter barley. They found two QTLs that were consistent between environments and years, 2H at 16.0 cM and 7H at 91.12 cM.

Using genetically defined mapping populations of barley, Mickelson et al. (82) conducted a detailed analysis of QTLs associated with N storage and remobilization and identified a number

of QTLs associated with NUE traits. Unfortunately, owing to lack of consensus markers on this map, and several mapping inconsistencies, only a few loci associated with NUE were identified. For ΔN (the difference between the N acquired by the plant prior to anthesis and the N in the harvest plant product) they found three QTLs on chromosomes 5H and 6H that explained 37.4% of the variation. In barley, Kindu et al. (73) found that although it was possible to detect QTLs for NUE-related traits in two separate years, the QTL analysis of NUE and its components mainly indicated inconsistent results across N levels and years, in line with low correlations among environments for a given trait. However, under the different conditions and between years there were two QTLs for NUEg (NUE of grains), NUEg, and NHI that consistently mapped to chromosomes 2H and 3H.

In wheat and barley, there is one group of QTLs that affects both NUE and GPC (64, 123, 124). These loci, known as *Gpc-B1*, affect N reallocation in these plants, resulting in significant differences in NUE and N allocation within the plant. Near-isolines with *Gpc-B1* in Australian varieties showed increased GPC without negative impact on grain yield (44). However, as noted above, GPC has a significant effect on bread quality (wheat) and malt quality (barley) and therefore must be evaluated with this in mind. To improve GPC without any penalty in grain yield is challenging, but some QTLs, such as those detected on chromosomes 1B and 2A for GPC, may be potential candidates for this purpose.

THE CHALLENGE OF IDENTIFYING CANDIDATE GENES FROM MAPPING STUDIES

One of the key reasons for mapping genes is to use the information to identify the candidate genes that affect a trait and either track them (and the trait) with markers or clone the allele. In a meta-analysis, Quraishi et al. (102) identified conserved regions of the genome syntenic between maize, sorghum, rice, and *Brachypodium distachyon* that affect NUE and suggested that there may be evolutionarily conserved regions that exist for NUE within the genome of cereals. One of the conserved regions contained the GS and GOGAT genes, which Quraishi et al. (102) suggest shows a strong link to NUE. However, given the imprecision of these mapping studies, there are many other genes that also cosegregate, and the identification of genes that might affect NUE is still based on our knowledge of the gene's function. For example, the meta-QTL analysis by Quraishi et al. (102) revealed that phenology genes such as *Ppd*, *Vrn*, and a semidwarf gene *Rht* were highly correlated with the NUE-related traits, as described in other studies (60, 75), which indicates that it is essential to address phenology and developmental genes with care when we interpret QTL results. The subsequent attribution of the differences in phenotype to these particular genes is thus still uncertain. Other mapping studies suffer from the same problem, namely that the challenges of mapping the trait prevent any narrowing of the mapping interval to a region of the chromosome that contains a reasonable number of genes. For example, Gallais & Hirel (52) identified a number of chromosomal regions that segregated for traits associated with NUE. However, these regions represented more than 25% of the mapped maize genome. As with previous QTL analyses (66, 78, 94), Gallais & Hirel (52) demonstrated that many of the desired NUE traits cosegregated with GS genes, particularly Gln4 on chromosome 5. However, as there are multiple GS genes and the mapping intervals are large, it seems that it would be difficult not to find at least one GS gene linked to a chromosomal region that segregated for NUE.

Theoretical studies (74) and computer simulations (4, 11, 127) have demonstrated that, especially for small samples, estimates of the proportion of genotypic variance explained by a QTL can be severely inflated, regardless of the statistical method used. This has a number of consequences for identifying candidate genes or for marker-assisted selection, including attempts to replicate earlier findings in independent studies, because of the fact that the location and variation explained by that QTL are incorrect. Weightings given to individual marker-trait associations as components of selection indices are often severely biased and have a large sampling error, resulting in overrating the prospects of using marker-assisted selection. In more complex genomes, this makes the prospects of fine mapping and cloning of a particular QTL a utopian adventure. We recently mapped many of the putative candidate genes for NUE in barley and compared these with the regions of the barley linkage groups where QTLs for NUE had been identified. We found that many of the logical candidate genes did not map to a QTL associated with NUE (114a). The difficulty is that by identifying a region that contains putative candidate genes, many researchers have already preselected their genes of interest. Despite the success for genes involved in heavy metal tolerance, we are not aware of a case in which researchers have map-based cloned a NUE (specific) gene.

CONCLUSIONS

Although improving NUE in crop plants represents a significant research challenge, it is nevertheless an area of enormous importance. There have been significant improvements in nutrient management in both developed and developing countries, although further improvements need to be made (109). One novel approach would be to try to develop plants that can fix their own N biologically; the approaches to this are discussed in more detail in the sidebar, Beyond NUE: Toward the Genetics of N-Fixing Plants.

From a farmer's perspective, if given the choice between a high-yielding variety and an Nefficient variety, they invariably select the former, at least in the developed world. Therefore, our goal must be to decrease the optimum fertilizer rate required for a crop while continuing to increase yield. Certainly, nutrient-efficient crops cannot compete if they do not maintain yield, regardless of the source of the genetics (allelic variation or a GMO trait). To some degree, breeders already target this strategy, given that average grain yields have increased steadily in the United States since the early 1980s without increased application rates of N fertilizer (59). Therefore, as has been noted by many researchers, if breeders select for improved yield at a constant N, by definition, NUE improves (61). Ideally, the N response curve of the future varieties will not only show N efficiency but will also be N responsive (62). However, yields will reach a plateau even

BEYOND NUE: TOWARD THE GENETICS OF N-FIXING PLANTS

What if the cereal crops' need for applied N fertilizer was reduced or even eliminated by genetically engineering cereals to fix their own nitrogen using atmospheric N₂? Only diazotrophic bacteria can fix N₂ into biologically reactive ammonia, using ~9 to 20 *nif* genes encoding the enzyme (nitrogenase) and the unique cofactor (FeMoco), both essential for function. Nitrogenase is oxygen labile and needs plenty of ATP and reducing power to catalyze N₂ fixation. The idea of developing N₂-fixing plants is not new; however, the ability to develop them is new. Two genetic methods have been proposed: (*a*) engineer cereals to allow for diazotrophic symbiosis or (*b*) stably transform cereals with the *nif* genes needed to make a functional nitrogenase (10, 95). Nitrogenase needs protection from O₂ and access to high levels of ATP and reducing power. Organelles like chloroplasts and mitochondria could provide the right conditions for plant-made nitrogenase. Currently, stable genetic modification of chloroplasts is more technically advanced than genetic modification of mitochondria; however, mitochondria are still a viable choice for enzyme location by nuclear encoding the *nif* genes with a signal peptide sequence that directs the polypeptide into the mitochondria.

if NUE is maximized, as secondary limiting factors such as water, nutrient availability, and/or environmental conditions become relevant.

Many researchers have attempted to map NUE and components of NUE in different crops. However, the challenges have been the degree of phenotypic variation for this complex trait and the difficulty in getting reliable data from field trial studies (80). There are now better statistical tools for molecular mapping, and the need for more careful experimental design and replicate testing has been recognized (90, 119). Moreover, thanks to the decrease in cost of genotyping, genomic selection with whole-genome prediction models has become realistic for plant breeding (36). Although the prediction models and experimental designs need finer tuning (46), this approach, in theory, will enable us to select even for minor QTLs and accelerate the breeding process. However, the reality may well be that although we will be able to map specific QTLs for NUE, these are likely to represent genetic variation specific to the species and environmental conditions. It is extremely unlikely that there will be a single magic bullet in the shape of a single gene or even a single QTL for all crop species that will allow us to develop more nutrient-efficient crops.

SUMMARY POINTS

- There are two main incentives to improve NUE in crop plants so that they use less N fertilizer; one is economic gain and the other is N pollution reduction. Sutton et al. (115) estimated the societal damage costs of excess N (e.g., water and air quality) in Europe to be US \$91 to \$466 billion annually.
- There is a significant amount of genetic variation and phenotypic plasticity for NUE. Phenotype can be affected by N uptake and water availability, different macronutrients and micronutrients, and the environment.
- Two key components comprise NUE: NUPE and NUtE. In theory, improving NUE could be achieved by improving NUPE, NUtE, or both.
- 4. The importance of NUpE and NUtE to NUE varies considerably depending on how these traits are measured or derived, the level of N fertilizer applied, and the germplasm under evaluation. Significant improvements in NUE require coordinated improvements of both NUpE and NUtE, and it is important to understand the interactions between uptake and utilization.
- Physiological traits that may affect NUpE include root architecture and any other characteristic that impacts the roots' ability to extract available N from the soil.
- 6. Economically important traits are frequently polygenic; therefore, it is unlikely that a single genotype or RIL contains the necessary contrast in the relevant genes underpinning the trait(s). The ideal high-NUE genotype has both high genetic N efficiency and high N responsiveness.
- 7. The number of QTLs detected in a given study depends on a number of different factors, including the size and type of mapping population used, the trait being investigated, the number of environments used for phenotyping, the environmental conditions, and the genome coverage. The numerous genes, each with small effects in the trait, are much more challenging, as they usually cannot be investigated individually. Also, QTL mapping tends to be imprecise, so many other genes cosegregate, and the identification of genes that might affect NUE is still based on our knowledge of the gene's function.

FUTURE ISSUES

- Improving NUE in crop plants represents a significant research challenge, it is nevertheless an area of enormous importance. Although breeders and farmers seem to better appreciate a cultivar with better grain yield under the same fertilization condition, our goal should be to decrease the optimum fertilizer rate required for a crop while continuing to increase yield.
- 2. Researchers need to focus on defining and measuring NUE in a manner that is appropriate to producers. Measuring NUE is difficult, if not impossible, unless researchers use much more care in soil sampling and measuring total plant N.
- 3. We recommend that all analysis look at N efficiency and N responsiveness under a defined set of soil conditions.
- 4. Studying NUpE and root systems, especially in soil, is still difficult and so it is one area that remains relatively unexplored.
- 5. Of the two types of genetic mapping for NUE, association mapping or traditional segregation analysis in defined crosses, both have advantages and disadvantages. However, both approaches are limited in that breeders rarely use the allelic variation identified to develop superior lines, and it will likely prove more useful to identify lines with superior NUE among existing commercial varieties.
- 6. Analysis of mapping populations in a manner that will yield useful data requires multisite and multiyear trials. Funding studies that do not provide the resources to conduct proper trials will result in data that are largely useless. Further, researchers should try to take advantage of mapping populations and association populations that have been well studied, as the greater the number of field trials a population has been tested under, the more useful the data set.
- 7. Researchers need to quantify more clearly the economic and environmental benefits of reducing N applications, and these quantifications need to be used in developing appropriate N applications policies.

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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