

19 Physiological and Molecular Interventions for Improving Nitrogen-Use Efficiency in Maize

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19.1 Introduction

N is an essential element for plant vegetative as well as reproductive growth. It is an important constituent in most biomolecules such as DNA, RNA, proteins and enzymes. Owing to the critical role of N in crop development and hence yield, nitrogenous fertilizers are usually utilized by farmers in the field. Although, up to some extent, these fertilizers are very helpful for increasing crop yield, excessive use of fertilizers is harmful for the environment and the ecosystem, on one hand, and increases the cost of cultivation, on the other. It was reported that the nitrogen-use efficiency (NUE) of cereal crops, including *Zea mays* (maize), does not exceed beyond 40–50% and the remaining 50–60% of N either leaches into the soil or evaporates into the environment (Raun and Johnson, 1999; Tilman *et al.*, 2002; Galloway *et al.*, 2014). There are many adverse effects of excess nitrogenous fertilizers, such as:

1. Sometimes excessively applied nitrogenous fertilizer promotes the growth of non-native plants which compete with and suppress the

growth of native plants, thereby creating an ecological imbalance in that particular area.

2. Too much N also causes the depletion of other important nutrients, such as Ca, P, Mg, etc. When these nutrients deplete from the soil, other toxic nutrients like Al could accumulate.

3. Excess NO_3^- from soil leaches into underground waters and drinking of NO_3^- -contaminated water causes various diseases like blue baby syndrome or methaemoglobinaemia (Majumdar, 2003). Other than this, when this N enters the food chain through vegetables and fruits, it may cause thyroid disorders, cancers and neural tube defects (Ward *et al.*, 2018).

4. The excess N from fertilizer application may lead to the formation of N_2O in the atmosphere, which is a potent greenhouse gas resulting in depletion of the atmospheric O_3 layer, thereby causing global warming (Wuebbles, 2009; Galloway *et al.*, 2014).

5. Nitrogenous fertilizers are very popular among farmers but manufacturing of nitrogenous fertilizer is an energy-intensive process because natural gas is used as both reactant and heat source for the reaction. This also adds to the cost of fertilizer, which ultimately may create a

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burden on the farmer's income as well as the environment (Smith, 2002).

Maize is one of the major cereal crops with wide adaptability under diverse agroclimatic conditions. Among the cereals, maize has the highest genetic yield potential. Being a C_4 crop, water- and C-use efficiency of this crop are higher than for other cereals like wheat and rice. It is also a day-neutral crop and can be grown in any season. This crop has wide utilities as food, fodder and feed as well as industrial uses. Owing to its myriad uses and the ever-increasing global population, demand for maize is increasing continuously. Like high-yielding crop varieties, single-cross maize hybrids required high levels of N for optimum production. Thus, to meet the increased global demand and obtain high yield, as well as in view of the prevalence of N deficiency in most soils globally, farmers excessively use N-based chemical fertilizers. As mentioned above, plants can utilize only half of the applied N from fertilizer. Therefore, development of novel strategies and maize genotypes which can utilize externally applied N more efficiently would be of prime importance to reduce the cost of cultivation, environmental pollution and achieve sustainable agriculture.

19.2 Importance of Nitrogen in Plant Growth and Development

For efficient plant growth and production, balanced nutrition is essential. Proper nutrient management plays a key role in increased food grain production and quality. In this regard, N occupies a central role in plant metabolism as it is an essential component of chlorophyll, proteins, enzymes, nucleic acids, etc. (Table 19.1). N is the fourth most important component of any living organism after C, H and O. Up to 4% of plant dry matter is N. Chlorophyll is the molecule which captures light energy and converts it into carbohydrate by the process of photosynthesis. Being part of the chlorophyll, N directly affects photosynthetic capacity of the plant and ultimately yield. Low N affects chlorophyll content resulting in reduced flow of available photosynthates to the growing regions, affecting overall plant growth. Protein is the building block of any living entity and DNA contains all its genetic information, which is a key molecule for plant

Table 19.1. General information about nitrogen.

Name	Nitrogen
Discovered by	Daniel Rutherford in 1772
Availability in air	78–79% by weight
Classified as	Non-metallic/macro element
Colour	Colourless
Present as	N_2 , NO_2 , NH_3 , NH_4^+ , NO_3^- , $C-NH_2$
Plant uses	NH_4^+ , (NO_3^-) , $C-NH_2$
Losses by	Leaching, volatilization, crop removal, erosion, etc.
Deficiency symptom	Yellowing of older leaves (V-shaped yellowing)
Excess symptom	Extra green colour to leaves and succulent growth
Contribution in agriculture	Promotes yield and quality

survival. N affects crop yield by increasing leaf area, canopy structure, net assimilation rate and photosynthetic capacity of the plant. For getting higher yield, N fertilizer application is unavoidable and indispensable as it also affects grain weight and quality. Maximum leaf area along with total leaf biomass are major determinants of crop yield. A constituent of chlorophyll, N imparts dark green colour to leaves and promotes vegetative growth of other plant parts. In maize, zein is the main storage protein in the endosperm and globulin in the embryo. These play an important role in seed germination and initial plant vigour (Moose and Below, 2009). During early stage of development, N promotes growth of roots which in turn absorb other mineral nutrients like P, K, etc. Root branching pattern influences N uptake from soil as root is the point of entry of N into the plant. Absorbed N gets assimilated into amino acids in the root or leaf tissues (Moose and Below, 2009). Maximum N uptake and assimilation take place during early growth stage. However, N uptake continues briefly after flowering. Kernel number and size among yield-attributing traits are influenced maximally by N application in maize (John and Schmitt, 2008). Starch synthesis in the endosperm of maize seeds is greatly influenced by N.

Being a macronutrient, N plays myriad roles in cellular physiology. Low N availability leads to poor growth in plants (Hull and Liu, 2005), chlorosis, necrosis of leaves and disorder in many physiological and biochemical characteristics

(Bray *et al.*, 2000). Deficiency of N in maize causes reduced growth, chlorosis in leaves (changing of the green colour of leaves into yellow), especially V-shaped chlorosis of lower leaves, and restricted lateral bud growth (Chun, 2005). Deficiency appears first in the lower leaves because N is mobile in the plant and under deficient condition it moves to the younger leaves (Soltabayeva *et al.*, 2018). The anthesis–silking interval (ASI) is most sensitive to any stress, including N stress. ASI beyond 5 days drastically affects grain yield (Elings *et al.*, 1996). Recently, Yadava *et al.* (2020) studied the effect of N starvation in maize and observed reduced growth along with reductions in leaf chlorophyll content, total soluble proteins and total biomass accumulation.

Excess nitrogenous fertilizer is also harmful for plants as it imparts dark green colour in leaves, succulent vegetative growth over reproductive growth and less fruit quantity with less quality. Plants take up N from soil in the form of NO_3^- in aerobic soils and NH_4^+ in flooded wetland or acidic soils. NO_3^- is the most common absorbable form rather than NH_4^+ , which is less available in soil. Soil and climatic condition play an important role in N uptake and utilization.

19.3 What is Nitrogen-Use Efficiency and How to Manage It?

An essential nutrient for plant growth and development, N is unavailable in its most prevalent form as atmospheric N_2 . Plants instead depend upon combined, or fixed, forms of N such as NH_4^+ and NO_3^- , also competing with microbes for the limited N that is available in soil. Thus, N is a major limiting nutrient for most agricultural ecosystems. Therefore, for restoring soil fertility and increasing crop yield, inorganic nitrogenous fertilizer application has become the most common practice. The nitrogenous fertilizers contain relatively soluble forms of N, which makes the absorption and assimilation easy from soil (Reetz *et al.*, 2015). However, plants cannot utilize the complete exogenous N applied in the form of chemical fertilizers.

The term ‘nitrogen-use efficiency’ refers to grain yield per unit of supplied N (by soil and fertilizers) (Moll *et al.*, 1987). It has been estimated that since 1961, total N applied has increased by

a factor of 4.4 and total protein production has increased only by a factor of 3.1; thus in actual terms NUE has declined from 66 to 46% (Lassal-etta *et al.*, 2016). NUE is made up of two components: (i) nitrogen-uptake efficiency (NUpE), which is the efficiency of absorption/uptake of per unit of supplied N; and (ii) nitrogen-utilization efficiency (NUE), which is the efficiency of assimilation and remobilization of plant N to ultimately produce grain. In general, NUE of applied N includes recovery efficiency (RE), physiological efficiency (PE) and agronomic efficiency (AE) (Huggins and Pan, 2003). The RE of applied N reflects the efficiency of above-ground N uptake per unit of N applied. PE is the efficiency by which N in above-ground plant parts is converted to grain, and AE is grain yield per unit of N applied (Hirel *et al.*, 2007). Large genetic variability has been observed in maize for both uptake efficiency and utilization efficiency of N (Han *et al.*, 2015). High-yielding varieties of maize are not necessarily the best ones, when N supply is reduced, because most of the breeding programmes are carried out under non-limiting N conditions. In intensive agriculture systems, where enormous amounts of nitrogenous fertilizer are applied, most of it is lost by leaching and runoff into the surface water or groundwater and poses a serious threat to human health (Majumdar, 2003; Ward *et al.*, 2018).

Initially, it was reported that NUpE is more important for better yield, because a significant positive correlation was established between use efficiency and accumulation of N (Sinclari and Vadez, 2002). Eventually other findings reported that under N-limited condition, N utilization affects grain yield in a significant way. Moreover, NUE encourages more N uptake and accumulation; for example, a plant which has faster growth rate at early developmental stage will have a more structured root system which in turn will promote more N uptake. Therefore, both N uptake and N utilization are dependent on one another and their contribution for plant growth and yield cannot be described in isolation. NUE can be increased by improving either NUpE or NUE or both; however, due to variable soil and climatic conditions, it is difficult to quantify the actual amount of N available to or absorbed by plants.

Variation in the NUpE affects all phases of maize growth including the development, activity and senescence of leaves and the initiation,

growth and composition of ovules (Uhart and Andrade, 1995). In maize, primary N assimilation and mobilization occur simultaneously in source leaves and contribute almost equally to the supply of N to the developing ear (Hirel *et al.*, 2007). It is therefore likely that the supply of N assimilates, their efficient translocation and their conversion during kernel formation contribute to the overall plant NUE (Canas *et al.*, 2009).

Low soil N is a major maize production constraint worldwide, particularly in the developing countries. The global demand for N fertilizers was 105 million tonnes in 2017 and is predicted to reach 111.5 million tonnes by 2022 (FAO, 2019). Maize is an N-responsive crop with higher demand for N fertilizers. NUE of maize varies from 25 to 50% (Tilman *et al.*, 2002). This suggests that wide cultivation of maize is associated with significant contamination of the environment due to N loss. Hence, high NUE should be a major breeding emphasis. Significant interaction of genotype and N fertilizer application has been reported in maize by a number of studies (Tollenaar *et al.*, 1997; Presterl *et al.*, 2002; Gallais and Coque, 2005; Haegele *et al.*, 2013; Mastrodomenico *et al.*, 2018). This interaction may be due to differential uptake of N applied or differential utilization of N. The former is important under conditions of high N input, while the latter is relevant under conditions of low N input (Gallais and Hirel, 2004). The complex nature of soil, microorganism and plant interaction makes selection of genotypes for high NUE difficult (Gallais and Coque, 2005). Screening a large number of maize genotypes first under hydroponic conditions followed by evaluation under field conditions could be a better strategy for identifying N-use-efficient maize genotypes.

19.4 Traits Influencing Nitrogen-Uptake Efficiency

NUpE is regulated by plant demand and affected by various factors (Ismande and Touraine, 1994) as described below.

19.4.1 Root system architecture

Roots, being the underground part of a plant, help in nutrient uptake and absorption. N is a

mobile element in soil as well as in plants. In soil it moves by mass flow. Under N-sufficient conditions with adequate water supply, plant nutrient uptake remains unaffected by root morphology and size. However, under water scarcity, length and spatial distribution of roots play an important role for nutrient accumulation (Sinclair and Vadez, 2002). It has been reported that N-efficient genotypes allocate relatively more N to roots at early growth stages of the plant (Niu *et al.*, 2007). Root senescence is also an important feature for nutrient uptake. It has been reported that major N uptake for grain happens post anthesis stage. Therefore, a plant staying green at later maturation stage to promote root growth is a desirable feature to enhance NUpE (Chun *et al.*, 2005; Yadava *et al.*, 2019).

19.4.2 Root nitrogen transporter system

In primary roots, the apical section is involved in NO_3^- sensing and signalling, and the basal section in NO_3^- acquisition. However, root tips exhibit higher capacity to absorb N and this capacity is dependent on plant age (Sorgonà *et al.*, 2011). N is absorbed by specific transporters located on root cell membranes, which may have high or low affinity. The high- and low- affinity nitrate transporters (NRTs) play a major role in NO_3^- transport when soil N concentration is low and high, respectively. In higher plants, nitrate transporters are classified mainly into two families, namely: (i) the nitrate transporter 1/peptide transporter (NRT1/PTR) family (NPF); and (ii) the NRT2 family, also called the major facilitator superfamily (Léran *et al.*, 2014). The availability of maize genome sequence has improved our understanding of how N uptake is carried out under both N-sufficient and N-deficient conditions. Garnett *et al.* (2013) reported that NO_3^- uptake capacity in maize plants after 40 days of leaf emergence is correlated with *ZmNRT2.1* and *ZmNRT2.2* genes encoding two high-affinity transporters. Yu *et al.* (2014) reported that expression of *ZmNRT2.1* and *ZmNRT2.2* was enhanced and inhibited in the roots of maize plants under high and low NO_3^- , respectively. Recently, it has been found that two maize NPF6

transporters, ZmNPF6.4 and ZmNPF6.6, are permeable to both NO_3^- and Cl^- and the latter transporter has selectivity for substrate when roots are exposed to high NO_3^- (Wen *et al.*, 2017). Different *NRTs* exhibit differential expression patterns during the plant life cycle and under different levels of NO_3^- concentration (Garnett *et al.*, 2013). For instance, increased expression level of many high-affinity *NRT2s* has been reported in *Arabidopsis*, maize and *Triticum aestivum* (wheat) under N deficiency, which in turn increased NO_3^- uptake (Okamoto *et al.*, 2003; Garnett *et al.*, 2013, 2015; Buchner and Hawkesford, 2014).

In soil, the concentration of NH_4^+ is much lower than that of NO_3^- and plants also prefer NO_3^- over NO_2^- . In higher plants, NH_4^+ is taken up by transporters of the ammonium transporter/methylammonium permease/Rhesus (AMT/MEP/Rh) family, located on root cell membranes, that show different cellular distribution and substrate affinities (Yuan *et al.*, 2007). Two ammonium transporters, ZmAMT1.1 and ZmAMT1.3, located in rhizodermis have been reported as a high-affinity transport system.

19.4.3 Interaction with microorganisms

Arbuscular mycorrhizal fungi (AMF) are symbiotic fungi which enter the root cortical cells and form arbuscules. This symbiotic association has been shown to improve nutrient uptake in plants. Therefore, for improving NUE, developing varieties with efficient symbiosis with AMF could be one of the strategies (Verzeaux *et al.*, 2017). Another possible approach could be to exploit the potential of various N-fixing bacteria such as diazotrophs which have the ability to colonize the roots of cereals (Parnell *et al.*, 2016). These N-fixing bacteria do not form root nodules as rhizobium, but they colonize the root surface and sometimes enter root tissue and provide sufficient amount of N to the plant. They are sold as biofertilizers or phytostimulators (Kuan *et al.*, 2016). Some other plant growth-promoting bacteria (PGPB) have also been reported to release hormone for root development, thus increasing nutrient acquisition (Cassán and Diaz-Zorita, 2016).

19.5 Traits Influencing Nitrogen-Utilization Efficiency

19.5.1 Nitrate assimilation

After the uptake of NO_3^- , it is generally transported to shoots for further assimilation. In the cytosol of leaf mesophyll cells, this NO_3^- is converted into NO_2^- by the enzyme nitrate reductase (NR; EC 1.6.6.1). Further, the enzyme nitrite reductase (NiR; EC 1.7.7.1) catalyses the reduction of NO_2^- into NH_4^+ in the plastids of these cells. Wang and Loussaert (2015) have shown that overexpression of *yeast nitrate transporter (YNT1)* in maize driven by root-preferred promoter led to yield improvement in the transgenic *YNT1*-overexpressing lines. Subsequently, irrespective of their origin, NH_4^+ ions (both synthesized via NO_3^- reduction and absorbed) are incorporated into the amino acid (organic form) glutamine and glutamate via assimilation primarily by glutamine synthetase (GS; EC 6.3.1.2) and sometimes by ferredoxin-dependent glutamate synthase (Fd-GOGAT; EC 1.4.7.1), also known as the GS/GOGAT cycle (Krapp, 2015). The generated glutamine and glutamate are used as amino group donors for most of the other amino acids, which are further incorporated into proteins and nucleic acids directly or transported through the phloem stream providing organic N to developing organs. In both C_3 and C_4 plants, various isoenzymes of GS and GOGAT are present which are located in different cellular compartments. Luo *et al.* (2015) reported that gene encoding GS1 isoenzymes coincides with a quantitative trait locus (QTL) for low N tolerance. That study confirmed that GS plays an important role in NUtE. It has also been reported that some variability exists in long-distance transport of amino acids in phloem (Yesbergenova-Cuny *et al.*, 2016), especially in the concentrations of amino acids and their translocation in phloem tissue for improving grain yield. Although amino acid translocation to developing sink organs and utilization play an important role, most of the studies have focused on vegetative organs such as root and shoot. The developing ear and tassel also play an important role in improving NUtE (Seebauer *et al.*, 2004; Liao *et al.*, 2012; Pan *et al.*, 2015).

19.5.2 Canopy photosynthesis per unit of nitrogen

It is well-established fact that foliar N content is the major determinant for leaf photosynthesis. Being a C₄ plant, maize has a much higher NUTe than most of the C₃ cereal plants. The best strategy for efficient N use is to absorb as well as utilize the whole N for grain yield. It has been reported that if N concentration in vegetative organs is the same, N-efficient maize hybrids still have greater photosynthetic rate at kernel-filling stage resulting in a higher number of kernels per ear (Chen *et al.*, 2006). One possible explanation for this behaviour might be that there is strong positive feedback on photosynthetic rate from the sink requirement in N-efficient cultivars. So, it can be concluded that for higher NUTe, it is important to develop more active reproductive sink units.

19.6 Identification and Use of Quantitative Trait Loci Related to Nitrogen-Use Efficiency

A QTL is a locus (section of DNA) that correlates with variation of a quantitative trait in the phenotype of a population of organisms. QTLs are mapped by identifying which molecular markers correlate with an observed trait. As NUE is defined as the production of grain yield per unit of N from soil and fertilizers, NUE must be a multi-gene/QTL-controlled trait. Numerous studies suggest that NUE and its related physiological traits such as N accumulation and re-translocation are mainly controlled by additive gene effects (Pollmer *et al.*, 1979; Below, 1997; Chen *et al.*, 2003). Therefore, identifying QTLs linked to NUE is a promising way for genetic improvement of NUE. The first maize NUE trait-related QTLs were reported by Agrama *et al.* (1999). In most of the studies in which QTL mapping was performed using recombinant inbred lines (RILs) (Table 19.2), the size of the characterized chromosomal regions was generally between 5 and 30 cM depending on the size of the population of RILs and on the measured agronomic and phenotypic trait (Gallais and Hirel, 2004; Jansen *et al.*, 2015). Agrama *et al.* (1999) found significant differences in QTLs

controlling grain yield and its components under high and low N supply, and the contribution of these QTLs to the phenotypes is between 11.8% (grain weight) and 42.1% (yield). Later studies have shown coincidences between QTLs and traits related to NUE which are controlled by specific genes involved in N uptake, metabolism and remobilization. Bertin and Gallais (2001) found QTLs related to NUE at high rather than low N supply, possibly because plant response to N is higher at high N inputs. They further reported that on chromosome 1 the region near to *bnlg1643* locus may be involved in grain yield determination under optimal N fertility, probably through efficient plant N uptake. Moreover, at the end of chromosome 6, locus *umc1653* plays a role in the adaptation to N-stress conditions through efficient grain filling (Coque and Gallais, 2006). Further, QTLs for root architecture and GS activity may be important for grain yield both under N-sufficient and N-deficient conditions. Analysing coincidences between QTLs for agronomic and physiological traits and key genes of N uptake and metabolism could be useful to identify genes/QTLs involved in the variations in NUE. By this means, one QTL for leaf GS1 enzyme activity has been shown to be coincident with a QTL for yield, one QTL for 1000-kernel weight was coincident with the *Gln3* (*gln1-4*) locus and two QTLs for 1000-kernel weight and yield were coincident with the *Gln4* (*gln1-3*) locus (Hirel *et al.*, 2001; Gallais and Hirel, 2004). Based on this information, *gln1-3* and *gln1-4* mutants, as well as the *gln1-3/gln1-4* double mutant, were isolated and analysed. The *gln1-4* phenotype displayed reduced kernel size and *gln1-3* reduced kernel number, with both phenotypes displayed in the *gln1-3/gln1-4* double mutant. All three mutants have not produced any kernels when grown under the N-limiting conditions (Hirel *et al.*, 2007). Moreover, when *gln1-3* gene was over-expressed constitutively in the leaves, kernel number increased by 30%, indicating that *gln1-3* plays a major role in kernel yield (Martin *et al.*, 2006). Based on these results, the GS locus on chromosome 5 appeared to be a good candidate gene which can, at least partially, explain the variation in NUE (Gallais and Hirel, 2004). These studies highlighted the possibility of increasing maize yield by optimizing N metabolic traits. Considering the importance of root traits in

Table 19.2. List of cloned/fine-mapped and important QTLs for NUE and associated traits in maize.

Study no.	QTLs/loci mapped ^a	Mapping population	Cross	Genotyping markers ^b	Chromosome or bin no.	Reference
1.	Two joint QTLs having additive effects across environments for GY per plant and NUE, as well as for biomass and N harvest index	RIL	B100 × LP2	SSR, SNP	1, 9	Mandolino <i>et al.</i> (2018)
2.	Five stable, low-N stress-specific QTLs	RIL	178 × K12	SSR	2.07, 2.03/2.04, 4.00/4.01, 5.02/5.03, 8.05/8.06	He <i>et al.</i> (2018)
3.	Five important QTL clusters in which QTLs for NUE and root system architecture-related traits coincided	RIL	Ye478 × Wu312	SSR	1.04, 2.04, 3.04, 3.05/3.06, 6.07/6.08	Li <i>et al.</i> (2015)
4.	A major QTL for AARL under low N co-localizes with QTLs previously reported for GY and N uptake	RIL	Z3 × 87-1	SSR	1	Liu <i>et al.</i> (2008)
5.	Eight QTL clusters in which QTLs for root architecture and traits related to N uptake, N remobilization and GY yield coincide positively	RILs	F-2 × Io	RFLP, SSR	2.6, 3.7, 4.2, 4.6, 5.1, 5.4, 5.5, 5.6	Coque <i>et al.</i> (2008)
6.	Two QTL clusters having QTLs for N remobilization and leaf senescence				6.4, 7.4	
7.	Genomic locus playing role in adaptation to N-stress conditions through efficient grain filling	RILs	F-2 × Io	SSR	6	Coque and Gallais (2006)
8.	One locus in which QTLs for CY, GS and NR enzyme activity, and NO ₃ ⁻ content coincide	RILs	F-2 × Io	RFLP	5 (<i>gln4</i> locus)	Hirel <i>et al.</i> (2001)
9.	Six QTLs involved in expression of GY components under low-N stress	F _{2:3}	B73 × G79	RFLP	1, 2, 7, 9, 10	Agrama <i>et al.</i> (1999)

^aGY, grain yield; AARL, average axial root length.

^bSSR, simple sequence repeat; SNP, single-nucleotide polymorphism; RFLP, restriction fragment length polymorphism.

NUE, various groups are working on QTLs related to maize and the most important QTLs were detected on chromosome bins 1.03, 1.06, 1.08, 2.03, 2.04, 7.02, 8.06 and 10.04 (Tuberosa *et al.*, 2003) In a meta-analysis study, Luo

et al. (2015) identified 21 consensus QTLs (cQTLs) strongly induced for low-N tolerance. He *et al.* (2018) identified five stable QTLs specific to low-N stress (*qPH5b*, *qLL4a*, *qSPAD-BEL2b*, *qSPADBEL8b* and *qGLN2a*) (Table 19.2). Out of

these five, four QTLs have been reported for first time and it is expected that candidate genes of these low-N stress-specific QTLs may be induced by low-N stress, which in turn might play a pivotal role in maize adaptation to N deficiency. Recently, map-based cloning was successfully utilized in tobacco to identify homologous genes involved in NUE (Edwards *et al.*, 2017). This approach has not been successful in maize so far. However, the recent approach of balanced multi-parental (MAGIC) populations (Dell'Acqua *et al.*, 2015) and ultra-high-density maps (Liu *et al.*, 2015; Su *et al.*, 2017) could provide powerful tools leading to higher power and definition in QTL mapping for complex traits such as NUE.

19.7 Identification of Nitrogen-Responsive Genes

A detailed understanding of NUE is necessary for optimizing fertilizer input without compromising crop yield. To understand and improve NUE, two major approaches can be adopted: (i) study the response of crop plants to N stress to identify the major genes and metabolic/biological processes affected by it; and (ii) utilization of natural or induced genetic variation in NUE. From microarray and other gene expression studies, various genes involved in C assimilation, N and C metabolism, NO_3^- assimilation (NR and GS) and NO_3^- transport (NRT1 and NRT2) were found differentially regulated under conditions of varied N application (Gutiérrez *et al.*, 2007; Amieur *et al.*, 2012; Schlüter *et al.*, 2012; Plett *et al.*, 2016; Jiang *et al.*, 2018; Yadava *et al.*, 2020). By transcriptomics and transgenic approaches, the roles of a set of genes (encoding transcription factors, nitrogen transporters and kinases) related to the regulation of N assimilation have also been investigated. Transcriptomics has played a key role in understanding NUE in plant systems, including *Arabidopsis*, rice, wheat and maize. Overexpression of *OsE-NOD93-1* (*early nodulin 93*) has been shown to lead to accumulation of higher concentrations of total amino acids and total N in roots (Sun *et al.*, 2014). Heterotrimeric G-proteins that regulate NUE were reported in rice (Kurai *et al.*, 2011; Sun *et al.*, 2014). Similar work has been carried out in *Arabidopsis* with Dof1 (DNA-binding

with one finger) (Table 19.3). In maize, by integrating meta-analysis and large-scale gene expression data, Luo *et al.* (2015) mined 30 candidate low-N stress tolerance genes and a further 12 most important maize orthologues were identified by *in silico* analyses of genes with known functions in NUE in model plants. Apart from genes, micro RNAs (miRNAs) responsive to NO_3^- stress have also been identified (Fisher *et al.*, 2013). In *Arabidopsis*, miR393/AFB3 has been shown as a unique N-responsive module controlling root system architecture in response to external and internal N availability (Vidal *et al.*, 2010). The repression of miR528a/b and miR169i/j/k in maize roots under NO_3^- stress suggested their role in integrating NO_3^- signals into root developmental changes (Trevisan *et al.*, 2012). Although significant progress has been made in the identification and characterization of genes and miRNAs playing critical roles in N uptake, translocation and homeostasis in *Arabidopsis* and rice, only a few studies are available in maize pertaining to the same. The potential candidate genes/miRNAs playing a pivotal role in NUE in *Arabidopsis* and rice (Table 19.3) or their maize orthologues might be utilized and explored for improving NUE in maize.

19.8 Nitrogen Signalling and Transduction for Improving Nitrogen-Use Efficiency

Signal transduction for any physiological process involves sensing and processing of stimuli. In the case of NUE in maize, Dof1 plays an important role. Dof1 is a transcription factor, a member of the DNA binding with one finger (Dof) family. It is unique to plants and facilitates expression of a range of genes associated with organic acid metabolism (Yanagisawa, 2004). The overexpression of *Dof1* in *Arabidopsis* under the control of a maize *pyruvate phosphate dikinase* (PPDK) promoter resulted in increased concentration of amino acids, especially glutamine, under low-N stress conditions, which in turn helped the transgenic plants to tolerate N stress (Yanagisawa, 2004). However, Cavalari *et al.* (2007) reported that Dof1 does not play a major role in the control of N or C metabolism in maize. Recently, Peña *et al.* (2017) have shown that

Table 19.3. List of key genes validated for improving N uptake and utilization in various crop plants that may be potentially useful for improving NUE in maize.

Gene name; source	Gene function	Crop engineered	Overexpression/silencing phenotype	Reference
Alanine aminotransferase (<i>AlaAT</i>); barley (<i>Hordeum vulgare</i>)	Involved in both C and N metabolism; catalyses the reversible reaction (by transfer of an amino group) converting alanine and 2-oxoglutarate to glutamate and pyruvate, and vice versa	<i>Brassica napus</i> spp. <i>oleifera</i> <i>O. sativa</i>	Overexpression using root-specific promoter resulted in significant increase in root biomass and NUE in transgenic lines	Good <i>et al.</i> (2007); Shrawat <i>et al.</i> (2008)
<i>AlaAT</i> ; from barley, mouse (both cytoplasmic and mitochondrial isoforms) and <i>Pyrococcus furiosus</i>		<i>Arabidopsis</i>	Overexpression of different variants of <i>AlaAT</i> conferred diverse NUE phenotypes under different external conditions	McAllister and Good (2015)
<i>Dof1</i> ; maize (<i>Zea mays</i>)	Regulates expression of genes encoding enzymes for C-skeleton production (e.g. upregulation of PEPC) and thereby modulates C/N network	<i>Arabidopsis</i> <i>Solanum tuberosum</i>	Overexpression resulted in enhanced N and amino acid contents (i.e. N assimilation) and increased plant growth under low-N conditions	Yanagisawa (2004)
		<i>O. sativa</i>	Overexpression resulted in increased C and N assimilation under low-N conditions	Kurai <i>et al.</i> (2011)
		<i>Triticum aestivum</i> <i>Sorghum bicolor</i>	Constitutive overexpression resulted in reduction in photosynthesis, plant height and biomass in transgenic lines, while tissue-specific expression under <i>rbcS1</i> (Rubisco subunit 1) promoter resulted in increased biomass and yield	Peña <i>et al.</i> (2017)
<i>NRT2.3b</i> ; rice (<i>Oryza sativa</i>)	NO_3^- uptake, enhances the pH-buffering capacity of the plant	<i>O. sativa</i>	Constitutive overexpression resulted in improved plant growth, yield and NUE by 40% in transgenic lines	Fan <i>et al.</i> (2016)
<i>NPF6.5 (NRT1.1B)</i> ; <i>O. sativa</i>	NO_3^- uptake and subsequent root-to-shoot transportation, NO_3^- signalling	<i>O. sativa</i>	Overexpression significantly improved NUE and yield	Hu <i>et al.</i> (2015)

Continued

Table 19.3. Continued.

Gene name; source	Gene function	Crop engineered	Overexpression/silencing phenotype	Reference
<i>NRT2.1</i> ; <i>O. sativa</i>	Interacts with OsNAR2.1 and plays a role in NO_3^- transport	<i>O. sativa</i>	Constitutive overexpression (using <i>ubiquitin</i> promoter) resulted in decreased NUE, while overexpression under native promoter resulted in increased NUE	Chen et al. (2016)
<i>NLP6</i> and <i>NLP8</i> ; <i>Z. mays</i>	NO_3^- signalling and metabolism, induction of NO_3^- -responsive genes	<i>Arabidopsis</i>	Overexpression restored NO_3^- signalling and assimilation in <i>nlp7</i> mutant, increased biomass and yield	Cao et al. (2017)
<i>ENOD93-1</i> ; <i>Oryza sativa</i>	Encodes the early nodulin 93 protein and expressed at high levels in roots, especially at the panicle emergence stage, localized in mitochondria; exact role not known	<i>O. sativa</i>	Overexpression resulted in higher accumulation of total N and amino acids in roots, increased shoot dry biomass and seed yield under N-limiting condition	Sun et al. (2014)

PEPC, phosphoenolpyruvate carboxylase.

overexpression of the maize *Dof1* in wheat led to an improvement in growth and productivity. These studies in wheat and *Arabidopsis* lead to the conclusion that *Dof1* transcription factor is an interesting candidate for increasing nutrient-use efficiency and yield potential of cereals. Other than *Dof1*, some regulatory genes such as *NLP7*, *PHR1* and protein kinase *AtCIP8* are key elements in regulating NO_3^- response in *Arabidopsis* (Castaings *et al.*, 2009). Nine *NLP* genes were identified in maize; out of them *ZmNLP6* and *ZmNLP8* regulate NO_3^- signalling in *Arabidopsis* and were able to increase plant biomass and yield when overexpressed in the model species (Cao *et al.*, 2017). In spite of studies related to identification of NUE-related QTLs and genes, the molecular mechanism governing NUE in cereals, including maize, is not fully understood. Therefore, more work is required in this field for better understanding of NO_3^- signalling so that this knowledge can be used for genetic manipulation and transformation for better yield results.

19.9 Conclusion

NUE is the key to sustainable agriculture in the coming future. Over the past few years, remarkable progress has been achieved in identifying molecular targets for improvement of NUE in various crops including maize. Various important genes having crucial roles in root development, N assimilation, N uptake and utilization, N signalling and sensing can be used for improving NUE. As discussed above, NUE is a complex phenomenon. So, neither conventional breeding

nor the transgenic approach alone is sufficient to increase the NUE. Soil N status and the plant's inherent nature towards N uptake and utilization are also important. For improving NUE in any field crop, a holistic approach which include soil management, agronomic practices, conventional breeding as well as transgenic approaches with specific transgenes will help in maintaining crop yield with minimum N input and will ensure environmentally friendly crop production.

Roots play a key role in N uptake. In this regard, root response studies should be undertaken more systematically. Root being an underground system is inherently difficult to phenotype, but several high-throughput screening techniques may help breeders and physiologists effectively. Selection of genotypes recording efficient growth under low N is indicative of better root architecture. Hence, this should be a general strategy for selection of efficient genotypes. Transcriptome analyses have identified a large number of genetic elements influencing NUE. These can be used as biomarkers for better NUE. However, these need further validation in a wider number of genotypes.

Intensive research on molecular and genetic aspects of NUE has led to the identification of many new genes, QTLs and alleles that could be deployed to develop new genotypes. The future direction of the research efforts should be towards understanding the interaction of NUE-related genes with cellular small RNA flux and perturbing the system performance through metabolic engineering and genome editing techniques. It is expected that these efforts would ultimately lead to commercialization of new improved maize hybrids with high NUE in the near future.

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