

Chapter 5

Molecular Targets for Improvement of Crop Nitrogen Use Efficiency: Current and Emerging Options



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Introduction

Food security is closely linked to nutrient availability for cropping, whereas its sustainability is directly linked to nutrient use efficiency. This is particularly true for nitrogen, which is quantitatively the most important component of all fertilizers. By 2050, there will be 70% increase in the global food demand, as the world population will increase to over 9.7 billion (Yang et al. 2012; York et al. 2016; Chen and Liao 2017). Unfortunately, the average N-use efficiency (NUE) in crops is about 30%, and the unutilized reactive N species that accumulate in the environment cause water and air pollution affecting health, biodiversity, and climate change (Sutton et al. 2013; Zhang et al. 2015). While short-term improvement of NUE at the farm level can be done using better agronomic practices, slow release fertilizers etc., the inherent ability of the crop to take up the available N and use it efficiently for maximal yield and minimal loss has to be tackled biologically.

A major biological challenge is that our idea of yield itself may vary between grain, fruit, seed, flower, leaf, and tuber depending on the crop. Another biological challenge is that out of the several dozens of definitions of NUE, very few are biologically relevant, such as uptake and utilization efficiency (Pathak et al. 2011; Yu et al. 2016). It is also not uncommon for yield-centric researchers to project N responsiveness as NUE. For example, a cultivar that keeps responding to increasing doses of N-fertilizer with slightly higher yield is misinterpreted as N-use efficient, even if its yield differential may keep falling with increasing N, making it actually less efficient. Such approaches also often push biologists to search for NUE within the narrow genetic pool of high-yielding varieties, rather than trying to find the true extent of genetic diversity that exists for NUE.

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The identification of the biological avenues for crop improvement toward NUE is hampered by the incomplete characterization of its phenotype and genotype (Pathak et al. 2011; Sinha et al. 2018). This is extremely important to identify contrasting varieties or to rank all the available germplasm in the increasing or decreasing order of NUE, so as to benefit from the fast-growing genomic data for association mapping. Some of the phenotypic traits associated with NUE so far include root length/number/branching/density, (Morita et al. 1988; Yang et al. 2012; Steffens and Rasmussen 2016), dense and erect panicle in rice (Sun et al. 2014), onset of post-anthesis senescence, and plant height in wheat (Gaju et al. 2011). This chapter is primarily focused on the recent advances in the molecular approaches to improve NUE in plants through the identification of the genes involved in N response and NUE and their manipulation by various means.

Molecular Aspects of N Response for NUE

N is present in soil in the form of nitrate (NO_3^-) or ammonium (NH_4^+) in aerobic or flooded (anaerobic/acidic) conditions, respectively. A small portion of N can also be absorbed in the form of amino acids or as urea directly by plants with the help of specific transporters. They are mainly absorbed through the roots and translocated throughout the plant through xylem. N-compounds are also recycled and remobilized from internal stores or senescing tissues through the phloem to the sites of demand, such as for grain filling in cereals. The genes involved in all these processes of N uptake, assimilation, and remobilization are important for N-use efficiency, which makes it a complex, quantitative trait. On an organism-wide scale, N response encompasses many more genes/processes that may contribute to NUE, including C metabolism, redox metabolism, and root/shoot development (Fig. 5.1). The molecular biology of N response has been elaborated in several reviews (Pathak et al. 2008; Krapp et al. 2014; Li et al. 2017; Sinha et al. 2018). Therefore, the following sections deal with various molecular targets that have been explored toward improvement of N-use efficiency.

Genes/QTLs Identified for NUE

Marker-assisted genetic mapping has helped identify many genes/QTLs for plant height, panicle weight, and panicle number such as *GSI*, *DEP1*, *NADH-GOGAT* to improve NUE. At the same time, many other genes involved in N transportation, assimilation, signaling, and regulation have been successfully used to improve NUE in rice and other plants (Fig. 5.1). Over-expression of *OsNRT2.3b* improved nitrate-uptake capacity, C metabolism, grain yield and thereby NUE by 40%. In addition, it also enhanced the uptake capacity of P and Fe by maintaining pH homeostasis (Fan et al. 2016). Therefore, modulation of the expression of N transporters has a beneficial impact on the overall plant NUE. However, it has also

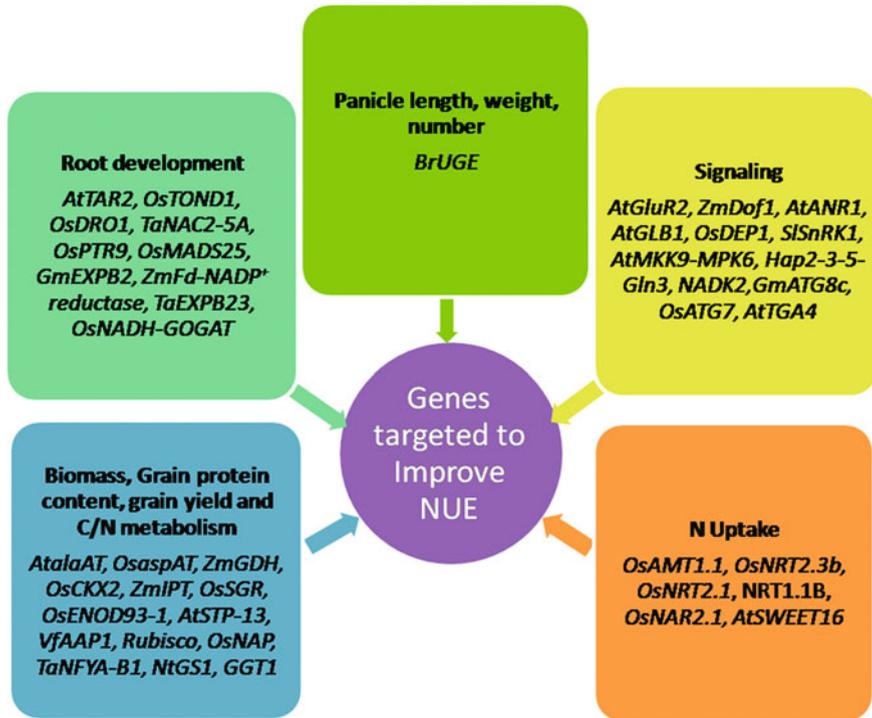


Fig. 5.1 N-responsive molecular targets of various physiological processes used to improve NUE. *AtAlaAT*: *A. thaliana* Alanine aminotransferase; *OsaspAT*: *O. sativa* Aspartate aminotransferase; *ZmGDH*: *Z. maeze* NADP-dependent glutamate dehydrogenase; *OsCKX2*: Cytokinin oxidase; *ZmIPT*: Cytokinin biosynthesis, *OsSGR*: Stay green, *OsENOD93-1*: Mitochondrial membrane protein; *AtSTP-13*: Hexose transporter, *VfAAP1*: *V. faba* Amino acid permease, Rubisco; *OsNAP*: NAC transcription factor, *TaNFYA-B1*: *T. aestivum* CCAAT-binding transcription factors, *NtGS1*: *N. tabacum* cytosolic glutamine synthetase, *AtGGT1*: glutamate: glyoxylate aminotransferase 1; *BrUGE*: *B. rapa* UDP-glucose 4-epimerase; *OsNADH-GOGAT*: NADH- dependent glutamate synthase; *AtTAR2, OsTOND1*: Tolerance Of Nitrogen Deficiency 1, *OsDRO1*: DEEPER ROOTING 1, *TaNAC2-5A, OsPTR9*: Peptide transporter/nitrate transporter, *OsMADS25, GmEXPB2*: *G. max* beta-expansin, *ZmFd-NADP⁺ reductase*: Ferredoxin NADP⁺ reductase, *TaEXPB23*; *AtSWEET1*: Sugars Will Eventually Be Exported Transporters; *AtGluR2*: Glutamate receptor; *AtGLB1*: PII regulatory protein; *OsDEP1*: (Dense and erect panicle 1) G protein γ subunit; *SlSnRK1*: *S. lycopersicum* sucrose non-fermenting-1-related protein kinase 1; *AtMKK9-MPK6*: Mitogen-activated protein kinase; *Hap2-3-5-Gln3*: Hap2-3-5 binding domain and Gln3 activation domain; *AtNADK2*: NAD kinase (Wang et al. 2012; Klemens et al. 2013; Alvarez et al. 2014; Rothstein et al. 2014; Dellero et al. 2015; Wada et al. 2015; Abdula et al. 2016; Li et al. 2016; Chen and Liao 2017; Wan et al. 2017)

been reported that an increased N input may delay flowering time and consequent yield losses especially in high-latitude regions where late-season temperatures hamper grain filling (Li et al. 2017). Transgenic approaches using many other genes involved in N metabolism have also been implicated to improve NUE such as glutamate dehydrogenase (*GDH*), aspartate aminotransferase (*AspAT*), and

asparagine synthetase (*AS*). NLP proteins have been recently reported to increase crop yield by improving plant biomass under both N-rich and poor soil conditions (Xu et al. 2016).

Molecular Manipulation of Root System Architecture for NUE

In the past two decades, scientific community has established a strong basis to target root system architecture as an approach to improve NUE (Forde 2014; Fan et al. 2017; Li et al. 2017). Root system is comprised of embryonic (primary and seminal roots in Arabidopsis and cereals, respectively) and post-embryonic roots (lateral roots in Arabidopsis; lateral, brace, and crown roots in cereals). Studies carried out in maize helped us to understand the advantages of “steep, cheap, and deep” root morphology to absorb water and nutrients from soil (Lynch 2013). Long and thick primary roots help plants to acquire N from the deeper horizon, while fewer and longer lateral roots with steep root growth angles not only decreases the metabolic cost but also help in exploring greater volume of soil.

Numerous signaling mechanisms are involved in the adjustment of root development to heterogeneous N environments. Studies on the molecular control of N-responsive root development have been mainly carried out in Arabidopsis, though various homologs of the genes involved have also been found in rice and other plants (Forde 2014; Shahzad and Amtmann 2017). A summary of N-responsive regulators of root system architecture is provided in Table 5.1. Arabidopsis shows root adjustment towards different levels and forms of N in the surrounding rhizosphere with the help of various signaling molecules. This includes regulation of lateral root initiation in the xylem pole pericycle cells by *CEP5* (C-terminally encoded peptide) in an auxin and N-dependent manner (Roberts et al. 2016); inhibition of lateral root emergence during systemic low N signal by *CLE* (*CLAVATA3/ESR*-related) gene family which binds to *CLAVATA1* (*CLV1* leucine-rich repeat receptor-like kinases (Araya et al. 2014, 2016; Okamoto et al. 2015); inhibition of primary root growth by *AFB3* (auxin receptors which are a part of the *SCF^{TIR1}/AFB E3* ubiquitin ligase complex) in the presence of nitrate and promotion of lateral root growth by *AFB3/NAC4/OBP4*-signaling module (Vidal et al. 2010, 2013). Recently, several miRNAs (miR167, miR393, miR160 and miR171) and N-responsive transcription factors have been reported to regulate root system morphology (Table 5.1) under various N conditions in Arabidopsis and rice (Vidal et al. 2010; Yan et al. 2014; Bellegarde et al. 2017; Chien et al. 2017; Gifford et al. 2017; Sun et al. 2017; Undurraga et al. 2017). Generally lateral roots are much more sensitive to the fluctuating nutritional conditions and their response depends on the degree of stress in the surrounding region. Low N deficiency tends to promote lateral root initiation but moderate to severe N deficiency hampers further root emergence and elongation. Root morphology is also determined by the ratio of $\text{NO}_3^-:\text{NH}_4^+$. High $\text{NO}_3^-:\text{NH}_4^+$ ratio showed positive effect on the lateral root length, whereas low ratio has a contrary impact (Qin et al. 2017).

Table 5.1 N-responsive genes involved in the regulation of root system architecture

Gene/Protein name	Organism	Function	References
Tryptophan aminotransferase-related protein 2 (<i>TAR2</i>)	<i>Arabidopsis thaliana</i>	Maintenance of the root stem cell	Ma et al. (2014)
MADS-box transcription factor, Arabidopsis Nitrate Regulated1 (<i>ANR1</i>)	<i>Arabidopsis thaliana</i>	Root plasticity in response to NO_3^- . Promotes NRT1.1 dependent lateral root growth	Zhang and Forde (1998), Remans et al. (2006), Gan et al. (2012)
C-terminally encoded peptides (<i>CEPs</i>)	<i>Arabidopsis thaliana</i>	Act locally to inhibit lateral root initiation	Ohyama et al. (2008), Roberts et al. (2016)
(<i>CEPs</i>)	<i>Arabidopsis thaliana</i>	Acts as long-distance signaling molecule	Tabata et al. (2014)
CLAVATA3/Endosperm surrounding region-related peptides (<i>CLE</i>)	<i>Arabidopsis thaliana</i>	Overexpressed <i>CLE1</i> to 7 inhibit lateral root development	Araya et al. (2014), Araya et al. (2016), Okamoto et al. (2015)
MiRNA167/Auxin Response Factor (<i>ARF8</i>)	<i>Arabidopsis thaliana</i>	Balancing between initiation and emergence of lateral roots	Gifford et al. (2017)
NAM, ATAF, and CUC transcription factor	<i>Arabidopsis thaliana</i>	Regulates primary and lateral roots development	Vidal et al. (2013)
MiRNA393/AFB3	<i>Arabidopsis thaliana</i>	Regulate development of Primary and lateral roots	Vidal et al. (2010)
miR444a/ANR1	<i>Oryza sativa</i>	Reduces nitrate induced lateral root formation	Yan et al. (2014)
EL5, a plant-specific ATL Family E3 Ubiquitin ligase	<i>Oryza sativa</i>	Maintains the viability of root apical meristem	Mochizuki et al. (2014), Nishizawa et al. (2015)
Arabidopsis plasma membrane H^+ -ATPase isoform 2(<i>AHA2</i>)	<i>Arabidopsis thaliana</i>	Promotes primary and lateral root development	Mlodzinska et al. (2015)
<i>OsMADS25</i>	<i>Oryza sativa</i>	Promotes lateral and primary root development	Yu et al. (2015)
NAM, ATAF, and CUC transcription factor (<i>TaNAC2-5A</i>)	<i>Triticum aestivum</i>	Promotes root growth	He et al. (2015)
NUCLEAR FACTOR Y (<i>TaNFYA-B1</i>)	<i>Triticum aestivum</i>	Stimulates root development	Qu et al. (2015)
MADS-Box Transcription Factor (<i>GmNMHC5</i>)	<i>Glycine max</i>	Promoted lateral root not primary root.	Liu et al. (2015)
Nitrate assimilation-related component 1 (<i>OsNAR2.1</i>)	<i>Oryza sativa</i>	Lateral root formation	Huang et al. (2015)
MEKK1 kinase	<i>Arabidopsis thaliana</i>	Inhibit primary root growth and increased lateral root	Forde et al. (2013)
PHOSPHATE 1 (<i>PHO1</i>) and Root System Architecture 1 (<i>RSA1</i>)	<i>Arabidopsis thaliana</i>	Control root allometry	Rosas et al. (2013)

(continued)

Table 5.1 (continued)

Gene/Protein name	Organism	Function	References
MADs-box gene <i>AGL21</i>	<i>Arabidopsis thaliana</i>	Positively regulated lateral root development	Yu et al. (2014)
ABA-insensitive 2 (<i>ABI2</i>), calcineurin-like protein (CBL)-interacting protein kinase (<i>CIPK23</i>)	<i>Arabidopsis thaliana</i>	Inhibits lateral root development	Ho et al. (2009)
β -GLUCOSIDASE1 (<i>BGI</i>)	<i>Arabidopsis thaliana</i>	Lateral root development	Ondzighi-Assoume et al. (2016)

N Transporters as Targets to Improve NUE

Understanding the molecular mechanism of N uptake and its regulation is of great significance toward the improvement of NUE. Nitrogen is taken up from soil mainly in the form of nitrate (NO_3^-), ammonium (NH_4^+), amino acids or peptides, and urea with the help of substrate-specific transporters. Most of these transporters mediate active transport depending on the proton gradient across plasma membrane except few which act as channels and mediate passive transport of solutes. These transporters are classified into low affinity transport systems (LATS) and high affinity transport systems (HATS), as well as in terms of constitutive or inducible. LATS function at a relatively higher concentration of N (>0.5 mM) and have larger K_m values (5 mM). On the other hand, HATS mediate transport at low N concentration (0.2–0.5 mM) and have smaller K_m values (of about 50 μM). Analysis of tissue-specific expression under varying concentration of N is very important for an efficient N uptake and therefore determining crop yield (Li et al. 2017).

Five main families of nitrate transporters are present in plants: nitrate transporter 1/peptide transporter/nitrate peptide transporter family (*NRT1/PTR/NPF*), *NRT2/*nitrate nitrite porter (*NRT2/NNP*), chloride channels (*CLCs*), slow anion channel-associated 1 homolog 3 (*SLAC1/SLAH*), and aluminum-activated malate transporters (*ALMT*) (Li et al. 2017).

Many ammonium transporters (*AMTs*) have also been targeted to improve NUE by analyzing the phenotypic changes of specific overexpressing or mutant lines. *AMTs* belong to the *AMT/MEP/Rhesus* transporter family, which are highly conserved in bacteria, fungi, and plants with more than 700 homologs in bacteria and plants. In *Arabidopsis*, there are 6 *AMTs* and rice genome has 12 *AMTs* which have been classified into two subfamilies: *OsAMT1* and *OsAMT2* (Li et al. 2017; Xuan et al. 2017). The activities of these transporters are also controlled by phosphorylation, thereby preventing the accumulation of NH_4^+ to toxic levels within the plant system.

Urea uptake and metabolism within the plant and its evaluation as a target for NUE has not received requisite attention, despite the fact that most of Asian agriculture depends on urea fertilization. Urea transport occurs in plants through

five different types of urea transporters, out of which DUR3 type have high affinity and others have low affinity. DUR3 is a 1 urea/1H⁺ symporter, whereas the low affinity urea transporters (tonoplast intrinsic protein, *TIP*) act as channels and are pH independent (Reddy and Ulaganathan 2015). Gene expression of DUR3-type transporters is controlled by ammonia, nitrate, and urea.

Nitrate and ammonium transporters are also sensitive to the pH changes of the rhizosphere, the apoplast, or the cytoplasm, as exemplified by modulation in the activity of *AtNPF6.3/AtCHL1/AtNRT1.1* and *OsNRT2.3b*. Similarly, water also affects N uptake and under the condition of drought stress, plants activate specific signaling pathways to overcome reduction in the N uptake. N starvation-induced basic leucine zipper (bZIP) transcription factor gene, *AtTGA4*, cytokinin synthesis gene isopentenyltransferase (*IPT*), and nodule inception-like 7 protein (*NLP7*) along with *NITRATE REGULATORY GENE2 (NRG2)* are reported to regulate the process of N uptake under these conditions.

Other regulators of the N transporters include transcription factors (TF) such as MADS-box TF *ANR1*, LOB Domain-Containing proteins (*LBD37/38/39*), Nin like proteins (*NLP6*, *NLP7*), Hypersensitivity to Low Pi-Elicited Primary Root Shortening 1 (*HRS1*), TGACG Sequence-specific Binding Protein 1 (*TGA1/4*), Squamosa Promoter Binding Protein-Like 9 (*SPL9*), Auxin Signaling F-Box 3 (*AFB3*), Nitrate Regulatory Gene (*NRG2*), Teosinte Branched 1/Cycloidea/Proliferating Cell Factor 20 (*TCP20*), GATA transcription factor, High Nitrogen Insensitive 9 (*HNI9*), shoot-derived peptide signals such as bZIPTF, HY5, root-derived peptide signals such as CEP and CLE, and miRNAs such as miR393 and miR169a (Marchive et al. 2013; Chien et al. 2017; Xuan et al. 2017).

Apart from these transporters, roots also release exudates in the form of ions, organic compounds, and enzymes to improve nutrient acquisition efficiency (Chen and Liao 2017). Symbiotic association with arbuscular mycorrhizal fungi (AMF) also enables plants such as rice, maize, wheat, and soybean to acquire diffusible nutrients and fixed carbon beyond the rhizosphere and at the same time also reduces the inefficient use of applied N to the soil. It improves the N availability in the rhizosphere through varying the composition of rhizobial microbial community. Recently, Verzeaux et al. (2017) reported improved NUE in wheat by AMF-assisted increased N uptake and accumulation.

Components of N Sensing and Signaling as Targets to Improve NUE

Transcriptomic studies carried out in Arabidopsis, rice, maize, and several other plant species have provided ample support to the fact that N in the form of either nitrate, ammonium, nitric oxide or nitrogen metabolites (L-Glutamate) plays pivotal role in controlling many biological processes in plants, such as root development, crop yield, seed dormancy, flowering time, and leaf development (Wang et al. 2004; Forde et al. 2013; Sun et al. 2016; O'Brien et al. 2016; Noguero and

Lacombe 2016). During this, N mainly acts as a signaling molecule to regulate the expression of genes involved in nutrient transport, metabolism, glycolysis, gluconeogenesis, hormonal activities, etc., in both roots and shoots (Chakraborty and Raghuram 2011). Genes involved in these processes include transcription factors (MADS-Box Transcription Factor), phosphoenolpyruvate carboxylase, Gln synthetase, Asn synthetase, tryptophan amino transferase, ribosomal proteins, initiation factors and many more (Calatrava et al. 2017; Okumoto and Versaw 2017; Liu et al. 2017; Undurraga et al. 2017). Therefore, the knowledge of sensing and signaling components will further enhance our ability to develop improved crop variety (Table 5.2). For example, *NRT1.1* and *NRT2.1* sense changes in N concentration occurring in the external medium and initiate Ca^{2+} -mediated signaling cascade involving phospholipase C (*PLC*). *CHLI/NPF6.3/NRT1.1* acts as a dual affinity nitrate transceptor and therefore have the ability to sense both high and low concentrations of N. This property is dependent on the phosphorylation status which is under the tight control of CBL-interacting protein kinase23 (*CIPK23*) (Ho et al. 2009; Bouguyon et al. 2015; Riveras et al. 2015; Undurraga et al. 2017).

Plastid localized PII proteins in plants interact with N-acetyl-L-glutamate kinase (*NAGK*) and acetyl-CoA carboxylase to promote arginine synthesis and fatty acid synthesis, respectively. Glutamine binds to the C-terminal extension of PII proteins to enhance its ability to form complex with *NAGK* (Gent and Forde 2017).

Through the work carried out in yeast, target of rapamycin (TOR) was identified. In budding yeast, it is found to participate in signaling pathway including nutrient and hormonal signaling and then passing the information to downstream effectors. Plant genomes also have homologs of mammalian or yeast *TORC1* complex. Activity of TOR and sucrose non-fermenting 1 (Snf1) kinase (*SnRK1* in plants) complement with one another to maintain C/N homeostasis under different environmental conditions by regulating several biologically important processes such as photosynthesis, tricarboxylic-acid cycle, and N assimilation by mainly controlling protein synthesis (Dobrenel et al. 2016; Sesma et al. 2017). Similarly, general amino acid control non-derepressible 2 (*GCN2*) kinases also plays a very important role in controlling protein synthesis by causing phosphorylation of eIF2 α initiation factor under N starvation. In Arabidopsis, there are 20 ionotropic glutamate-like receptors (*iGLR*) and 24 in rice which have important functional role in stomatal closure, root branching, and maintenance of primary root meristem (Weiland et al. 2014; Gent and Forde 2017).

Nitrogen requirements of crops are fulfilled by the legumes by the process of nodulation by symbiotic relationship with N-fixing bacteria. The availability of genetic mutants has enabled to carry out transcriptomic studies to find out the factors controlling nodulation. Generally, nodulation is promoted under low N supply and excess of N supply has a negative impact on the number of nodules formed. A number of mobile signaling molecules such as CLE peptides, TOO MUCH LOVE (*TML*), receptor-like kinases, *CORYNE* and *CLAVATA2*, *CEPs*, COMPACT ROOT ARCHITECTURE2 (*CRA2*), nodule inception protein

Table 5.2 Potential targets to improve N sensing and signaling toward NUE

Gene/Protein Name	Organism	Function
PII protein	<i>Arabidopsis thaliana</i>	Nitrogen sensing
PII protein	<i>Arabidopsis thaliana</i>	Maintains plant C-N balance
PII protein	<i>Arabidopsis thaliana</i>	Arginine biosynthesis
TOR signaling pathway	<i>Arabidopsis thaliana</i>	Positive regulator of protein synthesis and a negative regulator of protein turnover
GCN2 protein kinase pathway	<i>Arabidopsis thaliana</i>	Phosphorylates translation initiation factor in response to uncharged tRNAs
Glutamate receptors	<i>Arabidopsis thaliana</i>	Act as amino acid gated Ca ²⁺ channels
<i>NRT2.1</i>	<i>Arabidopsis thaliana</i>	long-distance transport of N
NRT1.1/AtNPF6.3	<i>Arabidopsis thaliana</i>	Transceptor for N
CLE (CLAVATA3/ESR-related) peptides and CLAVATA1 (CLV1) kinase	<i>Arabidopsis thaliana</i>	Expansion of roots in N-dependent manner
ELONGATED HYPOCOTYL5 (HY5) and a bZIP TF	<i>Arabidopsis thaliana</i>	Mobile signal mediates nitrate uptake
NF1 kinase	Wheat	Involved in signaling
2A PHOSPHATASE ASSOCIATED PROTEIN OF 46 KDa (<i>TAP46</i>)	<i>Arabidopsis thaliana</i>	Downstream effector of TOR protein

(*NIN*), *NIN*-like proteins (*NLP*), and miR172-EARLY NODULIN40 (*ENOD40*) module regulate nodulation (Murray et al. 2016).

In plants, nitric oxide plays a very important role in regulating many biological processes including seed germination, root development, senescence, plant immunity, and abiotic stress by controlling the expression of many regulatory components (Calatrava et al. 2017). Ammonium is also found to induce the expression of genes involved in N metabolism (*PEPC*, Gln synthetase, and Asn synthetase) and transport (Amino Acid Permease, *AAP1*) (Wang et al. 2004). It also affects ammonium uptake, assimilation, hormonal balance, and root system architecture by altering cytosolic pH and post-translational modification of proteins involved in these processes (Liu and Wirén 2017). In *Arabidopsis*, ammonia is sensed by an ammonium transporter (*AtAMT1;1*) whose activity is modulated by the calcineurin-B-like-interacting protein kinases (CIPK) proteins by phosphorylation (Xuan et al. 2017).

Molecular Targets Among the Genes of N Assimilation and Remobilization

Nitrate taken up inside the root cells is first reduced by nitrate reductase (*NIA*) to nitrite and then to ammonium by nitrite reductase (*NiR*). Two *NIA* genes exist in Arabidopsis and three in rice. Subsequently, nitrite moves into the plastid and is then metabolized into ammonium by the glutamine synthetase/glutamate synthase (*GS/GOGAT*) cycle. Ammonium is further incorporated into amino acids. This process of amino acid formation depends on the availability of photosynthates. *GS* is a very important enzyme for N assimilation and remobilization and there are two isoforms of the enzyme: *GS1* that carries out primary ammonium assimilation in roots or re-assimilation of ammonium in leaves and *GS2* that carries out assimilation of ammonium in chloroplast. Three-to-five members of *GS* have been found in different plant species; for example, there are three in rice. Depending on the electron donor specificity, there are two types of *GOGAT*, viz. ferredoxin-dependent (*Fd-GOGAT*) and NADH-dependent (*NADH-GOGAT*). *GLU1* and *GLU2* are two *Fd-GOGATs* and *GLT* is the only *NADH-GOGAT* gene present in the genome of Arabidopsis. Similarly, rice genome encodes one *Fd-GOGAT* and two *NADH-GOGAT*.

Single gene transgenics overexpressing the genes of primary N assimilation (*NR*, *NiR* and plastidic *GS*, *GOGAT*) did not radically improve NUE (Pathak et al. 2008, 2011; Krapp et al. 2014; Sinha et al. 2018). This was expected in a quantitative, multigenic trait like NUE, which involves the coordinated expression of several genes including, but not limited to N-assimilation. This made regulatory targets more attractive than metabolic targets, but the inability to find specific nitrate response elements common to all N-responsive genes has delayed progress in this direction (Das et al. 2007; Pathak et al. 2009). Circadian clock master regulator, CIRCADIAN CLOCK-ASSOCIATED 1 (*CCA1*) also controls the expression of genes involved in N assimilation and thereby establishes a link between N metabolism and circadian clock (Gutiérrez et al. 2008). Kinases and phosphatases are also involved in the regulation of expression of genes coding for N assimilatory enzymes such as *NR*, *NiR*, *GS2*, and *Fd-GOGAT* (Undurraga et al. 2017). Another level of control of metabolism is carried out by transcription factors (*Dof*, *NLP7*, *GATA*), N metabolites (glutamine and glutamate), and miRNAs (Chien et al. 2017; Zuluaga et al. 2017). miR5640 targets phosphoenolpyruvate carboxylase (*PEPC*) which plays a very important role in maintaining C/N balance. The expression of *PEPC* and several other enzymes of tricarboxylic-acid cycle are also under the control of *Dof1* (*DNA BINDING WITH ONE FINGER*) TF (He et al. 2015). Castaings et al. (2009) reported the role *NLP7* protein in N assimilation and sensing. All *NLP* proteins can bind nitrate-responsive cis-element *NRE* and mediate nitrate-dependent gene expression (Marchive et al. 2013; Xu et al. 2016; Yu et al. 2016) and improve C/N balance under both N-sufficient and N-deficient conditions. On the one hand, proper N assimilation is required for chloroplast development, synthesis of chlorophyll, and proteins such as Rubisco ((ribulose-1,5-bisphosphate

carboxylase/oxygenase and *PEPC*), whereas on the other hand, C assimilation provides energy source for N metabolism in the form of reducing equivalents (Ferredoxin and NADH) and C skeleton for synthesis of amino acids.

Remobilization of nitrate from source (leaves) to sink (developing parts) is also a significant determinant of NUE as it recycles organic N to the seeds during the grain-filling stage and therefore determines the crop yield. Leaf senescence is the underlying phenomenon of nutrient remobilization which facilitates the recycling of photosynthates to the developing seeds. Autophagy promotes senescence of aging plant parts. Several senescence-associated genes (*ATG* and metacaspases) are expressed at different stages of plant senescence (Havé et al. 2016). This process involves the participation of tissue-specific transporters which replenishes the N requirement during reproductive stage of plant development. Several reports suggest the regulators of this process, such as nitrogen limitation adaptation (*NLA*), which control the expression of *AtNRT1.7* by protein ubiquitination pathway. However, *NLA* is itself under the control of miRNA827 (Liu et al. 2016). Analysis of rice *GOGAT* mutant leads to the identification of another protein, viz. ferredoxin-dependent glutamate synthase (*OsFd-GOGAT*), to play a role in this process (Zeng et al. 2016). *Fd-GOGAT* plays a role in ammonium recycling by photorespiration.

Various Approaches to Identify More QTLs Associated with NUE

Modern technologies have improved our ability to study the regulation at the level of gene expression. These techniques include TARGET (Transient Transformation System for Genome-Wide Transcription Factor Target Discovery) and ChIP-Seq (Bargmann et al. 2013; Marchive et al. 2013). A major challenge in crop improvement for nitrogen use efficiency (NUE) is that neither the phenotypic traits nor the genes/alleles determining NUE are clearly defined. Therefore, in this scenario it is very necessary to work with chemist and use analogs of different N sources and then carry out the phenotypic screening for NUE-related genes/loci. For example, by using chlorate, the toxic analog of nitrate, *OsNRT1.1B*, was identified as a critical QTL contributing to NUE divergence between rice subspecies (Hu et al. 2015). Similar strategy had earlier lead to the identification of several regulators of N assimilation in fungi and algae such as *NIT2*, *NIT4*, *AREA*, *NIRA*, and *NIT2* (Castaings et al. 2009). Application of the new high-throughput measuring techniques such as genome-wide association studies (GWAS) also enables us to identify genes/QTLs regulating NUE. For example, Gifford et al. (2013) grew 96 Arabidopsis accessions under two N regimes and studied root phenotypic traits and identified *JASMONATE RESPONSIVE 1 (JRI)* as one of the candidate genes. Similarly, *CALCIUM SENSOR RECEPTOR*, *PhzC*, *ROOT SYSTEM ARCHITECTURE 1*, and *PHOSPHATE 1* were discovered by high-throughput automated root image analysis

(Gifford et al. 2013; Rosas et al. 2013; Slovak et al. 2014). This technique helps us to study natural variations among different genotypes of a plant species and understand the complex regulatory mechanism behind NUE. Another such technique is semiautomated confocal microscopy with the help of which *KURZ UND KLEIN* (F-box family gene), was identified to play a significant role in root development (Li et al. 2017). Systems biology has also enabled us to identify novel interacting partners and further provides the missing knowledge about the components of signal transduction pathway of N sensing, signaling and metabolism (Gutiérrez et al. 2008; Vidal et al. 2013).

Conclusions

The last decade has witnessed tremendous progress in finding several molecular targets towards the improvement of N-use efficiency of plants. Several genes belonging to various processes have been identified including root development, N uptake, assimilation, and remobilization. In addition, genes involved in N sensing, signaling, and the regulation of the above processes have also emerged, including epigenetic regulation involving miRNA. While phenotype development has not kept pace with these developments, functional genomics and reverse genetics are opening newer opportunities for identification and validation of newer molecular targets. These developments strengthen the hope that improved crop varieties for NUE will become increasingly available for sustainable agriculture in the near future.

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