Title

Strategies for engineering improved nitrogen use efficiency in crop plants via redistribution and recycling of organic nitrogen

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Highlights

- Manipulation of ureide and amino acid transporters changes whole plant distribution of nitrogen and improves NUE
- Nitrogen from macromolecules can be recycled via selective autophagy pathways with greatest benefits under N stress
- Targeting alleles regulating plant responses to nitrogen may improve grain protein content

Abstract

Global use of nitrogen (N) fertilizers has increased sevenfold from 1960 to 1995 but much of the N applied is lost to the environment. Modifying the temporal and spatial distribution of organic N within the plant can lead to improved grain yield and/or grain protein content for the same or reduced N fertilizer inputs. Biotechnological approaches to modify whole plant distribution of amino acids and ureides has proven successful in several crop species. Manipulating selective autophagy pathways in crops has also improved N remobilization efficiency to sink tissues whilst the contribution of ribophagy, RNA and purine catabolism to N recycling in crops is still too early to foretell. Improved recycling and remobilization of N must exploit N-stress responsive transcriptional regulators, N-sensing or phloem-localized promotors and genetic variation for N-responsive traits.

Introduction

Nitrogen use efficiency (NUE), a measure of how the applied N fertilizer is used by the plant, has decreased globally in cereal crops from ~80% in 1960 to ~25% in 2000 due to increased annual global application without equivalent increases in global cereal production [1]. Nitrogen lost from agricultural systems can cause severe ecosystem damage from the eutrophication of terrestrial and aquatic ecosystems to the release of the ozone-depleting greenhouse gas nitrous oxide into the air. Agronomic and physiological studies performed in the field have led to improvements in NUE, notably via the use of legume cover crops and modifying the timing and placement of fertilizers. Additionally, genetic approaches have made use of diverse germplasm responses to external N supply and via QTL mapping of component traits related to NUE, they have identified candidate genes controlling photoperiod, dwarfing and vernalisation, and tillering responses to N [2,3].

The interactions between N, water and other nutrients complicates the assessment of NUE so it is often dissected into sub-component traits [4]. These traits include Nitrogen Uptake Efficiency (NUpE, the capacity of the plant to take up N), Nitrogen Utilization Efficiency (NUtE, the ability of the plant to convert N taken up into grain yield), and Nitrogen Remobilization Efficiency (NRE, the amount of N remobilized to the grain or crop component post-anthesis) [5]. Given that nitrate (NO_3^-) is the predominant N form available to and taken up by plants growing in well-aerated soils, the efficiency of the (NO_3^-) uptake systems and control of (NO_3^-) signaling are vital components of NUE. The accumulation of glutamine appears to have a negative feedback effect on the activity of high-affinity (NO_3^-) and ammonium (NH_4^+) transporters. However, some novel strategies to manipulate (NO_3^-) sensing, signaling and remobilization have overcome this limitation and led to improved NUE and yield [6-9]. These strategies did not, however, lead to improved grain protein concentration, which is an important factor in legume and cereal production.

Nitrate absorbed by plant roots is assimilated locally or after translocation to the shoot into organic N compounds in energy-intensive assimilation reactions (Figure 1). Manipulation of N utilization is one of the standard approaches to improve NUE. Over the decades, various physiological steps in N metabolism were dissected and genes involved were identified [10]. Among them, glutamine synthetase (GS), glutamine dehydrogenase (GDH), and alanine aminotransferase (ALAAT) were studied intensively in multiple species and showed some advantageous phenotypes such as increased biomass, seed yield and NUE [11,12].

In a secondary assimilation process, plants can use N derived from the catabolism of organic N macromolecules including nucleic acids and proteins, and their derivatives nucleotides, peptides and amino acids. Some of these compounds are remobilized to other plant organs after temporal N and carbon stress events. In later stages of development, the proteins in leaves are catabolized, releasing amino acids and other nitrogenous compounds for transport to reproductive and storage organs. In cereals, 50-90% of seed N is derived from these remobilized sources [12].

Biotechnological approaches to change the whole plant distribution of organic N metabolites, such as amino acids and ureides, have proven successful for the increase of NUE in several crop species [10,13]. Furthermore, manipulating selective autophagy pathways has also led to greater plant growth, N remobilization and, in some examples, improved seed yield [14]. However, there are still significant gaps in our knowledge that we will highlight in this review. Lastly, we suggest that combining tools to identify natural genetic variation with genome-editing techniques could have the greatest impact of all on improving NUE.

Recycling N from organic compounds

Recycling N from macromolecules via autophagy

Macromolecules are recycled and remobilized via selective autophagy pathways during nutrient starvation [14] and in the absence of stress. Autophagy involves assembly of a portion of the cytoplasm, which can include organelles, into a double-membrane vesicle (autophagosome), and delivery to the vacuole. This involves interaction with more than 40 AuTophaGy (ATG) genes in yeast, several of which have been identified and studied in plants [15]. Here we selectively present approaches taken to manipulate autophagy as related to plant N nutrition (Figure 1).

The loss of function *atg* Arabidopsis mutants are hypersensitive to N or C deficiency [16] and display an early leaf senescence phenotype under both optimal and N-poor conditions [17], and a lower N remobilization efficiency to the seeds [18] and a higher N to C ratio in their rosette leaves [14]. A similar sensitivity of the maize *atg12* mutant to N-starvation was evidenced with growth arrest at the seedling stage whilst mature plants displayed early leaf senescence and stunted ear development [19]. Heterologous expression of *SiATG8a* from foxtail millet under the control of a ubiquitin promotor improved the tolerance of rice to low N supply in terms of greater biomass and total N content of above-ground parts, however, the effect on grain yield was not determined [20]. In another study, overexpression of *MdATG18a* in apple enhanced leaf N content and expression of nitrate reductase

(*NIA2*), when grown under N starvation conditions [21]. In rice, under optimum conditions, overexpression of *OsATG8a* or *OsATG8b* lead to increased grain yield and enhanced N remobilization to the seeds [22,23]. The effect of ATG8 overexpression in N deficient conditions was investigated only with *OsATG8a*, and the findings show that, whilst N uptake efficiency was improved, the yield and NUE benefits seen at optimum N were no longer evidenced [23].

Although *ATG* genes are constitutively transcribed, their expression does increase in response to stresses including nitrogen starvation [24], and thereby transcriptional regulators are potential targets to enhance autophagy pathways. Wang et al. [24] demonstrated that the bZIP transcription factor, TGA9, activates *ATG* gene expression under both sucrose starvation and osmotic stress, yet its role in the regulation of autophagy in plants exposed to N stress conditions remains untested.

There is some early evidence that a selective autophagy pathway termed 'aggrephagy' exists in plants, shown by induction of degradation of protein aggregates under low nitrogen conditions in BY-2 tobacco cells and furthermore, *atg* mutants, which cannot deliver cargo to the vacuole, accumulated cytosolic protein aggregates (Figure 1) [15]. Mature ribosomes, rich in proteins and nucleotides, can also be targeted for degradation in a process called 'ribophagy', which is triggered by N starvation in yeast [25]. In plants, ribophagy involves the ubiquitin protease Ubp3/Bre5 complex, autophagy-like pathway, vacuolar lysis of ribosomal RNA (rRNA) [25] and the release of nucleosides (Figure 1). rRNA decay occurs in vacuoles by the activity of a non-specific endoribonuclease *RNS2* with AtRNS2 mutants having a longer half-life of rRNA [26]. RNA represents an important source of N for recycling, accounting for ~2.5% of the total N pool in wheat seedlings with depletion of half of this under N starvation [27]. It is vital to first demonstrate that ribophagy and aggrephagy are induced under N starvation *in-planta*. Considering the importance of nucleotide homeostasis for cellular metabolic functions [28], there are possible detrimental effects of manipulating ribophagy on nucleoside/nucleotide homeostasis.

Recycling N from purine metabolism

Nodulated tropical and sub-tropical legumes typically use allantoin and allantoate, collectively referred to as ureides, as the main form of N translocated. The infected plant cells host the N_2-fixing form of the bacteria, where N_2 is reduced to ammonium (NH_4^+) before being assimilated into glutamine and amides in the plant cytosol. Glutamine is then used for *de novo* purine synthesis and is catabolized in a series of oxidative steps to produce the ureides that can be completely broken down to glyoxylate releasing CO_2 and NH_4^+ as byproducts to be utilized or remobilized [29]. Non-fixing soybean plants also synthesise

ureides where they contribute 1-10% of the total N that is transported in the xylem to young leaves [30]. Allantoin was also identified in non-legumes where it is rapidly catabolized under N stress with elevated levels of purine catabolic pathway transcripts [31] and proteins [32]. Allantoin and its precursor xanthine were demonstrated to support photosynthesis and growth of wheat seedlings when supplied under N starvation [27]. Arabidopsis mutants of xanthine dehydrogenase (*Atxdh1*), allantoinase (*Ataln*) and allantoate amidohydrolase (*Ataah*) impaired in purine catabolism, display a NO_3^--dependent early senescence phenotype, reduced NUE and decreased fertility [32,33]. Overexpression of these genes may lead to improved NUE except for the complication of the opposing function of allantoin, which is to assist in plant recovery from drought and salt stress via downregulation of *ALN* [31]. Until the regulators of differential *ALN* expression are identified, altering whole plant distribution of the ureides, controlled by the xylem and phloem-loading ureide permeases (UPS) may be a safer option (Figure 1). The UPS transporters are important for plant growth and development with the *Atups1* and *Atups2* mutants displaying symptoms similar to N deficiency such as early flowering and reduced size at maturity [33].

Manipulating nitrogen remobilization to sink organs

Nitrogen moves from source organs (e.g. leaves) to sink organs (e.g. newer leaves, reproductive organs) as programmed. Movement fluctuates with the N status and developmental stage of the plant. When the N status is relatively low plants remobilize N more efficiently. On the other hands, plants tend to retain N in source organs when N is sufficient [34]. This negative feedback regulation of N remobilization can be a tweaking point to improve NRE, especially for crops favoured for higher protein content in the seed (e.g. wheat and soybean).

Ureide transport

The ureide allantoin is an effective N storage and transport form due to its high N:C ratio [31]. Manipulation of UPS, which moves allantoin/allantoate from roots to source leaves to sink leaves in leguminous and non-leguminous plant species alike, can lead to improved seed yield and protein content [30,35], Figure 1. For instance, overexpression of soybean *UPS* in nodulated soybean leads to increased export of ureides from the transgenic nodules, enhanced symbiotic N_2 fixation per nodule and increased seed yield [35]. Overexpression of the common bean *PvUPS1* in the phloem of non-nodulated soybean plants leads to increased source-to-sink transport of ureides and increased vegetative growth and seed number [30]. In another example, overexpression of the rice UPS1 by activation tagging (*OsUPS1-OE*) resulted in enhanced allantoin and total free amino acids accumulation in their panicles

[36]. There was no apparent negative impact of *OsUPS1-OE* at optimal N supply and the plants were larger with more tillers than wildtype plants when grown at sub-optimal N supply [36].

Amino acid and peptide transport

Amino acids are the dominant organic N form transported within a plant. Redistribution of amino acids can be dynamic, such as from root to shoot or vegetative to reproductive tissues. Many amino acid transporters have been identified for their specific roles [10,13,37]. It is noteworthy that some amino acid transporters are essential for plant growth. Rice lysine histidine transporter 1 (OsLHT1) is a key transporter for amino acid uptake and root to shoot translocation. *Oslht1* knockout rice lines displayed reduced biomass and grain yield compared to wildtype [38,39]. It would be intriguing to manipulate the expression of *LHT1* for the purpose of improving NUE [40]. There are many biotechnological approaches to manipulate amino acid and peptide transporters [10], here and in Figure 1 we highlight those with the most promise. Overexpressed rice lines of amino acid permease 1 (*AAP1*) displayed increased tiller number and grain yield [41]. In legumes, pea plants overexpressing *AAP1* had increased biomass under different N conditions [42]. OsAAP3 and OsAAP5 regulate tiller number and grain yield in both wildtype and overexpressed rice lines [43,44]. Downregulation of these genes by RNAi or CRISPR gene-editing technologies led to rice plants with increased tiller number and grain yield [43,44]. An amino acid transporter involved in phloem loading, AAP6, was found to regulate grain protein content in rice [45] and wheat [46]. Overexpressed soybean AAP6 plants showed high N accumulation even under N deficient condition [47]. Effects of overexpression of peptide transporters (PTRs) were also tested in rice for N use improvement. Rice *OsPTR6* overexpressed plants showed increased biomass and N uptake except when grown at high NH_4^+ provision [48], whereas *OsPTR9* overexpressed rice lines displayed increased biomass and grain yield [49]. Considering the large size of the PTR gene family, there are many more PTRs to be tested for NUE improvement in crops.

Exploiting natural variation

There is also great potential in studying natural variation in traits related to NUE and then using genome editing tools to mimic those variants in cultivated lines. For instance, in a sophisticated study by Liu et al. [3], the effect of N on tillering in diverse rice germplasm was assessed and a transcription factor *OsTCP19* which targets a tillering promoting gene, was identified. Genome editing of the non-beneficial allele, *OsTCP19-L*, led to a higher tillering response to N in the edited plant [3]. Considering that genetic variation and genome regions associated with grain protein content at varying rates of N supply are

known in major cereal crops [50], a similar strategy to that used by Liu et al. [3] can be employed to manipulate grain protein content.

A successful target for N remobilization is the grain protein content (GPC) locus *GPC-B1*, which was mapped, and the responsible gene *NAM-B1* (a NAC transcription factor) cloned in wheat [51]. Functional NAM-B1 enhances the remobilization of nutrients, including N, Zn, and Fe [51,52]. As the functional *GPC-B1* allele is rare in modern wheat varieties, *NAM-B1* has been incorporated in wheat breeding programs, providing traits of high grain protein content and quality without a concomitant yield penalty [53]. The homoeologous genes of *NAM-B1* (i.e. *NAM-A1*, and *NAM-D1*) in wheat and an orthologous gene in barley also showed similar functions [54-56]. As the NAM genes are transcription factors, there are opportunities to find other regulatory components that may serve as suitable targets for manipulating nutrient remobilization in crops [57].

Conclusion

There is the potential for great improvements in NUE via manipulation of whole plant distribution of organic N metabolites (Figure 1). The use of phloem-specific promotors (e.g. *proPvUPS1* [30]) to regulate long-distance N remobilization should be explored for both ureide and peptide transporters in cereal crops based on the positive results from these approaches in legumes. Improving the efficiency of N recycling from macromolecules in source leaves, particularly under conditions of N deficiency, can be achieved via manipulation of autophagy genes (ATGs) [23] and their corresponding transcription factors. Study of the pathway and regulation of ribophagy in plants and its contribution to nitrogen recycling under different N regimes is still in its infancy, and should first be better defined in model plants. Manipulating degradation of ribosomes/rRNA and purine metabolites (e.g. allantoin) may have unintended consequences by altering nucleotide pool homeostasis that is integral to many plant processes [28]. The precision required to maximize N remobilization may be assisted by internal molecular N sensors – the promotors from allantoinase (proALN, which is involved in ureide metabolism) and the ureide transporter, UPS1, are both highly responsive to N status in rice [58]. There is little known about the regulation of transporters involved in organic nitrogen remobilization by post-translational modification. It would be interesting to investigate whether their activity is also regulated by SUMOylation and phosphorylation as was shown for nitrate reductase, an important protein involved in nitrate assimilation (59). Additionally, studying natural variation in traits responsive to nitrogen and manipulating these using genome editing techniques has been largely under-utilized despite that the generation of marker-free crops with improved NUE is now possible. **Figures**

Figure 1. Schematic representation of gene-encoding targets and pathways of nitrogen utilization, recycling or remobilization. Transport of organic nitrogen from source to sink organs occurs in the phloem, however, phloem parenchyma localization of these proteins in crop plant species is not known for every example presented. Traits improved from various studies are summarized in purple boxes as shown; biomass (Bm), grain yield (GY), Nitrogen Use Efficiency (NUE), Grain Protein Content (GPC), Grain Nitrogen (GN), Nitrogen Uptake Efficiency (NupE), Root traits (R), N (total nitrogen content/concentration) under low nitrogen (LN) or sufficient nitrogen (SN). Rice is shown here by example, but improvements in many traits, were demonstrated in legume crops (nitrogen fixation [N_2 FIX] is specific to nodulated legumes). Ammonium (NH_4^+) remobilized from roots or older leaves, products of photorespiration, metabolites from amino acid recycling or from the complete oxidation of ureides can be assimilated into glutamine or into other amino acids via transamination reactions. Manipulation of amino acid transport via overexpression or reduced expression of genes encoding amino acid permeases (AAP) transporters has been successful to improve traits. There is early evidence that manipulating selective autophagy pathways via genes encoding AuTophaGy proteins (ATG) could improve nutrient remobilization. In brief, autophagy pathways involve: a. encapsulation of cytoplasmic components, organelles, ribonucleases or protein aggregates; b. autophagosome formation; and c. delivery of the autophagosome to the vacuole for lysis by proteases or ribonucleases. Vacuolar lysis of rRNA releases nucleosides that can be transported via equilibrative nucleoside transporters (ENT) [27,28]. Peptides and amino acids recycled from proteins are transported to developing organs via the activity of peptide transporters (PTR) and AAPs, respectively. Ureide permeases (UPS) transport allantoin and/or allantoate, products of purine nucleotide breakdown. Overexpression of UPS genes led to improved plant growth. Ureides and amino acids are mostly allocated to the xylem for movement to transpiring leaves, however, they can be transferred from the xylem to the phloem for supply to sink organs.

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