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Review

Advances in Understanding the Molecular Mechanisms and Potential Genetic Improvement for Nitrogen Use Efficiency in Barley

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Abstract: Nitrogen (N) fertilization plays an important role in crop production; however, excessive and inefficient use of N fertilizer is a global issue that incurs high production costs, pollutes the environment and increases the emission of greenhouse gases. To overcome these negative consequences, improving nitrogen use efficiency (NUE) would be a key factor for profitable crop production either by increasing yield or reducing fertilizer cost. In contrast to soil and crop management practices, understanding the molecular mechanisms in NUE and developing new varieties with improved NUE is more environmentally and economically friendly. In this review, we highlight the recent progress in understanding and improving nitrogen use efficiency in barley, with perspectives on the impact of N on plant morphology and agronomic performance, NUE and its components such as N uptake and utilization, QTLs and candidate genes controlling NUE, and new strategies for NUE improvement.

Keywords: nitrogen use efficiency; barley; candidate genes; QTL; genetic improvement

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1. Introduction

Soil nitrogen (N) availability usually limits plant yields such that large quantities of synthetic N fertilizers are applied to ensure maximum productivity. However, excessive N use is a significant issue around the world. For example, NPK fertilizer use in China increased from 0.73 million tons in 1961 to 54.16 million tons in 2015 [1,2]. The Food and Agriculture Organization of the United Nations estimated that N consumption would be around 119 million tons by 2020 with the increased population growth and demand for food [3]. Based on available data, N fertilizer demand is expected to increase by 1.2% per annum until 2022 [4]. Although high rates of N are applied, crop absorption is most likely 30%–50% [5]. The remaining N is leached into the environment and soil or lost through surface runoff and erosion. Consequently, N residues cause considerable adverse effects on the environment and human health by water, soil and air pollution. They contaminate groundwater, deplete the ozone layer and increase greenhouse gas levels (i.e., N_2O), causing global warming [6,7]. Thus, developing crop varieties with improved nitrogen use efficiency (NUE) that require fewer N inputs is economically and environmentally favourable to maintain the same or higher grain yields.

There are two major approaches to improving NUE, viz genotypic improvement through conventional breeding and genetic improvement through manipulating specific NUE-associated genes. Several studies have been undertaken to improve NUE in crops including rice, wheat and maize [8–11]. Starting from simple phenotypic screening through to advanced molecular techniques, crop performance under low N has been improved [12,13]. There are a few success stories for rice NUE improvement by genetic engineering [14–16]. For instance, overexpression of alanine aminotransferase in both rice and canola under a tissue-specific promoter increased yield under low N [14,17]. Similarly, the overexpression of nitrate transporters increased grain yield and NUE in rice under low N [18]. The outcomes of these experiments have shed light on the enhancement of crop NUE.

Barley is widely used for livestock feed and malting, and a small proportion is consumed as food. Due to its diploid nature, it is a good genetic model for other crops in the Triticeae family. Recent advances in barley NUE research have identified a few QTLs responsible for NUE and related traits [19,20]. However, most are limited by low genetic diversity and the small plant populations used. Indeed, the improvement in NUE in barley is in the early stages and needs further exploration. QTLs controlling NUE and associated genes in the model plant Arabidopsis and other cereal crops are useful for barley NUE research [21–24]. Therefore, identifying and understanding the genetic basis behind nitrogen use efficiency in barley and then altering the genes through genetic engineering may be a promising approach to improve NUE in barley.

2. Effect of N Fertilizers on Crop Growth and Yield

N plays an important role in the vegetative and reproductive development of crop plants. It is an essential nutrient in almost all stages of the growth cycle of crops for initiating early rapid growth, leaf development, stem extension, and increasing tiller numbers, grain size, grain protein content and, ultimately, yield [25,26]. It is present in the protein structure and chlorophyll, which, in turn, influence photosynthetic activity. High N accelerates the translocation of photosynthetic products from source to sink to increase yield [27]. In rice, yield increased by 16.6% due to an increase in productive tillers under high N supply [28]. The application of high rates of N produces higher yields by increasing major yield components such as tiller number, grain size, and grain number per spike in barley [29–31]. On the other hand, yield declines considerably under low N supply. In a study conducted on spring barley, yield declined by 70%–100% under low N compared to high N [29]. Low N stress causes slow growth and chlorosis, where leaf yellowing symptoms occur first in older leaves [26]. N-deficient leaves are narrow, small and erect which might die under severe stress. Eventually, it decreases photosynthesis and in the long-term results in reduced total production of photosynthate and grain yield.

During vegetative growth, plants uptake more N; thus, the shoots and roots incorporate a large quantity of N to increase biomass [32]. In wheat, total biomass, straw biomass and straw N content had a significant positive correlation with yield under N sufficient and deficient conditions [33]. During grain filling, 70%–90% of grain N is transported from internal reserves in vegetative organs [34]. The amount of N that remains in the grain is responsible for grain protein content, which determines grain quality [35–37].

3. Nitrogen Uptake, Assimilation and Use Efficiency in Crops

N absorption by plants comprises three main steps: uptake, assimilation and remobilization. N is naturally available from organic matter mineralization, biological N fixation, atmospheric N deposition, irrigation water and other organic sources such as farmyard manure [38]. In addition, inorganic N fertilizers are supplied externally to maximize productivity. Nitrogen is taken up in the form of ammonium or nitrate, depending on the soil conditions, by ammonium (AMT) and nitrate transport (NRT1/NRT2) systems, respectively [39]. Generally, NRT1 is the low-affinity transport system (LATS) and NRT2 is the high-affinity transport system (HATS). Of the NRT1 transporters, *AtNRT1.5* is involved in long-distance transport of nitrate from roots to shoots [40]. HATS is active when the external nitrate concentration is low [41]. The upregulated expression of NRT2.1, NRT2.2, NRT2.4 and NRT2.5 in Arabidopsis roots under N starvation is a good example of this [42]. Plant morphology and root characteristics mainly affect N uptake. In general, the root systems in low N soil develop better and extend deeper into the soil to enhance nitrogen uptake [43,44]. Nitrogen uptake also differs at different growth stages. For instance, plants uptake less N during reproductive crop development but facilitate N remobilization [45].

The absorbed inorganic N is converted into organic N compounds through primary and secondary assimilation [46]. Nitrate absorbed is first reduced to nitrite and then to ammonium by nitrate and nitrite reductases, respectively. The ammonium is assimilated in the chloroplast/plastids to amino acids by glutamine synthetase (GS) or glutamate synthase (GOGAT), which are further used for protein synthesis and the catalysis of biological pathways such as photosynthesis [47]. In addition to the GS/GOGAT cycle, some other enzymes including cytosolic asparagine synthetase, carbamoylphosphate synthase (CPSase) and glutamate dehydrogenase (GDH) are involved in ammonium assimilation [39,48]. N remobilization occurs during senescence through extensive degradation of proteins in older leaves to provide N to younger plant organs [39,49]. Studies conducted on *Arabidopsis thaliana* and *Brassica napus* revealed that N is remobilized to younger leaves during vegetative growth and seeds during reproductive growth [50,51]. Flag leaf senescence is responsible for N availability for grain filling in barley, wheat and maize [39].

Nitrogen use efficiency (NUE) can be defined in several ways, but the most common definition is grain yield per unit of N supplied (Table 1) [52]. This depends on two major components: Nitrogen Uptake Efficiency (NUpE) and Nitrogen Utilization Efficiency (NUtE) [52,53]. NUpE is the amount of N taken up by the plant per unit of N supplied whereas NUtE is the grain yield per unit of N taken up by the plant. Therefore, NUE is simply the product of NUpE and NUtE [52,54]. NUE is also described as NUEg, which is grain production per unit of N available, or as utilization index (UI), which is the absolute amount of biomass produced per unit of N. Environmental factors affect NUE, which include but are not limited to soil condition, fertilizer types, application timing, and the genotypic variability of the plant [53]. For rainfed wheat in India, topography, rainfall, and moisture availability affected NUE and grain yield [55]. Similar studies have been conducted to check the factors above controlling NUE using a wide range of other crops such as maize, vegetables and root crops [55]. Fertilizer applications and available soil N should be balanced to ensure that N is effectively used. However, more often, N is wasted due to low plant NUE. Thus, improving NUE is essential for cereal crops including barley, to minimize N loss, the negative impacts on the environment, and production costs.

Table 1. Definitions for nitrogen use efficiency (NUE) and its components [52].

Abbreviation	Term	Definition
NUE	N Use Efficiency	NUpE × NUtE = Yield/N supplied
NUpE	N Uptake Efficiency	NUp/N(soil + fertilizer) = Acquired N/N available
NUtE	N Utilization Efficiency	Yield/NUp
NUEg	N Use Efficiency Grain	Grain production/Available N
UI	Utilization Index	Total plant biomass/Total plant N

4. NUE Screening and Phenotyping

Preliminary screening of different crop genotypes is necessary to understand their performance under different N concentrations prior to any NUE improvement method. Initially, the yield was considered as the only trait related to NUE, thus stable yield performance under low N supply was a major approach for identifying N-use efficient genotypes. However, various research studies on cereal crops have revealed some other important traits, such as grain protein content, grain nitrogen content, grain weight, and shoot and root parameters (length, dry biomass, etc.) [19,21]. The relative performance of these agronomic traits is generally studied under low and normal N to identify NUE of plants. In rice, deeper roots, longer roots, and higher root length density and root oxidation activity are important traits screened for higher grain yield and NUE under low N conditions [56].

Field experiments are the most commonly used screening method [57], but these are difficult for NUE since they restrict the observation of root characteristics. In fields, N availability should be measured at multiple sites rather than merging a common value for the whole field because N in the soil can vary over very short distances. Therefore, pot and hydroponic experiments in growth chambers have been extensively conducted [12,58]. A comparison of all three screening methods revealed that the latter two approaches reduce environmental interference on genetic screening [29].

Several field experiments have been undertaken to screen barley NUE [29,57,59]. The experimental design (number of plots and replicates), soil N concentration, and geographic and climatic conditions play a key role in field trials [57]. A field trial conducted by [60], using 146 recombinant inbred lines (RILs) from Karl × Lewis in two replicate years identified several significant QTLs for N remobilization across barley chromosomes and several QTLs overlapped with other traits such as N metabolism. Similarly, screening of 224 spring barley accessions at three different locations in two replicate years identified 21 QTLs for thousand kernel weight, which is a major yield component and NUE attribute [61]. A Prisma × Apex barley RIL mapping population was used in pot experiments in two different years, which mapped 41 QTLs for 18 phenotypic traits under low N. Of these, 15 QTLs were responsible for NUE across six chromosomes except for chromosome 4H [20].

However, many studies have suggested that hydroponic experiments overcome the technical difficulties in root phenotyping in N uptake researches [12,62]. Hydroponics, using a nutrient solution as the cultivation medium instead of soil, facilitates the study of the N uptake mechanism and its impact on plant growth [63] with its easy observation of both root and shoot characteristics. Recently, a hydroponic experiment examined the shoot and root traits of five wheat genotypes at four different levels of N to identify high NUE genotypes [12]. Likewise, a hydroponic experiment on 82 Tibetan barley accessions investigated their performance under low N in terms of shoot and root dry biomass [64]. Ideally, performing all three methods together would give the most reliable, precise and comparable results when screening plant NUE.

5. QTL Mapping and the Major Loci Controlling NUE

Nitrogen use efficiency is a quantitative trait controlled by multiple genes [65]. Advances in molecular marker development, quantitative genetics and bioinformatics increase the possibility of identifying quantitative trait loci (QTLs) controlling NUE. QTLs for NUE have been identified in Arabidopsis and other cereals such as rice, wheat and maize [48,66–69]. Both agronomic traits such as grain yield, grain protein content, and grain weight [66,69,70], and NUE traits such as N remobilization efficiency, N content in the grain and N harvest index [20] have been used as indicators of NUE. In rice, four QTLs have been identified for grain nitrogen content and two QTLs for shoot nitrogen content under both low and normal N on chromosomes 8, 9 and 10 using 166 lines of RILs. In addition, two QTLs were identified on chromosomes 5 and 7 for harvest index and 1 QTL on chromosome 9 for physiological NUE under low N [71]. There are some other QTLs identified in rice for N response, grain yield response and physiological NUE [72]. Recently, significant QTLs have been detected for root NUE, shoot dry weight and grain yield from a wheat TN18×LM6 RIL population [73]. Thus, the studies conducted in rice, wheat and maize set a background for NUE research in barley [21,23,71,74,75].

Although a limited number of studies have been undertaken to identify QTLs controlling NUE under low N in barley, Table 2 summarises a list of major QTLs identified up to date. Fifteen significant QTLs were detected for NUE and its components in the barley Prisma × Apex population under low N [20]. Besides, a few genome-wide association studies have identified QTLs controlling yield, grain weight and grain protein content, which are key indicators of NUE [61,70,76]. However, the results have been inconsistent between studies and between experimental years due to the small mapping populations, low marker density, limited genetic diversity and environmental factors. It seems that QTL mapping to identify candidate genes for NUE is quite challenging. Therefore, it is important to use a large population size with substantial genetic diversity and to conduct multiple field/pot trials across several growing seasons with sufficient biological replicates to minimize these shortcomings and provide more reliable results.

Table 2. List of major quantitative trait loci (QTLs) related to NUE and NUE-related traits in barley.

Chr	QTL	Trait	Genes Co-Localized	Population	Parent with Positive Allele	Reference
1H	qYld	Yield	*HvIPT1*	Morex × Barke	Barke	[19]
	qYld	Yield	*HvIPT1*	Orria × Plaisant	Orria	[77]
	qGPC	Grain protein content	*HvCKX5*	Morex × Barke	Barke	[19,61]
	qGW	Grain weight				[76]
2H	qYld	Yield	*HvCKX7, HvGDH3*	Morex × Barke	Barke	[19]
	qYld	Yield	*HvPKABA7*			[70]
	qYld	Yield	*HvCKX7*	Multiple varieties	n/a	[78]
	qGPC	Grain protein content	*HvAMT1.2, HvGS3, HvGOX1, HvIPT2, HvGOX2, HvGOGAT2*	Morex × Barke	Barke	[19]
	qGPC	Grain protein content	*HvCIN2, HvAMT1.2 HvNAM-2, HvGOX1*	Lewis × Karl	Lewis	[60]
	qGPC	Grain protein content	*HvIPT2, HvGOX2, HvGOGAT2, HvPKABA5, HvAlaAT2-2, HvCIN2*	Barley CAP spring lines	n/a	[70]
	qNUEg	NUE of grains	-	Apex × Prisma	Prisma	[20]
	qNutEg	NUtE of grains	-	Apex × Prisma	Prisma	[20]
	qNHI	N harvest index of grains	-	Apex × Prisma	Prisma	[20]
3H	qYld	Yield	*HvCKX3*	Morex × Barke	Barke	[19]
	qYld	Yield	*HvASP4, HvCKX3*			[70]
	qNUEb	NUE of above-ground biomass	-	Apex × Prisma	Prisma	[20]
	qNupEb	NUpE of grains	-	Apex × Prisma	Prisma	[20]
4H	qGPC	Grain protein content	*HvCIN1, HvGS4*	Morex × Barke	Barke	[19]
	qGPC	Grain protein content	*HvCIN1*	Barley CAP spring lines	n/a	[70]
	qGPC	Grain protein content	*HvGS4*	Multiple varieties	n/a	[61]
	qGW	Grain weight	*HvGS4*	Morex × Barke	Barke	[19]
	qGW	Grain weight	*HvGS4*	615 UK barley genotypes	n/a	[76]
5H	qYld	Yield		Lewis × Karl	Lewis	[60]
	qGPC	Grain protein content	*HvPKABA6, HvFNR2*	Morex × Barke	Barke	[19,70]
				Multiple varieties	n/a	[61,70]
	qNUEb	NUE of above-ground biomass	-	Apex × Prisma	Prisma	[20]
	qNUEg	NUE of grains	-	Apex × Prisma	Prisma	[20]
6H	qYld	Yield		Morex × Barke	Barke	[19]
	qYld	Yield	*HvNR3, HvASP5*	Multiple varieties	n/a	[79]
				Lewis × Karl	Lewis	[60]
	qGPC	Grain protein content	*HvNR1, HvGS1*	Morex × Barke	Barke	[19]
	qGPC	Grain protein content	*HvNAM1*	Barley CAP spring lines	n/a	[70]
	qGPC	Grain protein content	*HvNAM1, HvNAR2.1, HvAMT1.1*	Lewis × Karl	Lewis	[60]
	qGHI	Harvest index		Apex × Prisma	Prisma	[20]

Table 2. *Cont.*

Chr	QTL	Trait	Genes Co-Localized	Population	Parent with Positive Allele	Reference
7H	qYld	Yield	*HvNRT2.7, HvLHT2, HvLHT3*	Morex × Barke	Barke	[19]
	qYld	Yield	*HvLHT2, HvLHT3*	Multiple varieties	n/a	[78]
	qYld	Yield	*HvNRT2.7*	Multiple varieties	n/a	[79]
	qGN	Grain N		Morex × Barke	Barke	[19]
	qNHI	N harvest index of grains	-	Apex × Prisma	Prisma	[20]

Cytokinin biosynthesis (IPT), Cytokinin oxidase (CKX), Glutamate dehydrogenase NAD(P)H (GDH), Sucrose non-fermenting-1-related (PKABA), Ammonium transporter (AMT), Glutamine synthetase (GS), Glycolate oxidase (GOX), Glutamate synthase (GOGAT), Cell wall invertase (CIN), NAM transcription factor (NAM), Alanine aminotransferase (AlaAT), Aspartate aminotransferase (ASP), Ferredoxin NAD(P)H reductase (FNR), Nitrate reductase (NR), NRT partner protein (NAR), Nitrate transporter (NRT), Lysine histidine transporter (LH), n/a (not available).

6. Functional Genes for NUE

Genetic and molecular mechanisms in NUE have been extensively investigated in rice and maize, which holds the potential to expand the knowledge to other cereals. As a result, a number of candidate genes and gene families have been identified from these studies to improve NUE [15,65]. Nitrate and ammonium transporters are one of the important functional genes identified. There are about 70 nitrate (NO_3^-) transporters in Arabidopsis and over 85 in rice that are supposed to be candidates for NUE improvement [48]. Overexpression of *OsNRT1.1* in rice under low N conditions in field increased grain yield per plant by 32%–50% and NUE by 38%–54% per plot through a significant increase in seed number per panicle and thousand grain weight whereas its mutations decreased the panicle size, seed setting rate and grain yield [15,80,81]. Similarly, overexpression of *OsNRT2.1*, *OsNRT2.3b* and *OsPTR9* in rice increased NUE, grain yield and plant growth [18].

The 12 ammonium transporters (AMT) in rice differ in their roles in N uptake and transportation at different growth stages. Transcript levels of most *OsAMTs* are significantly upregulated in response to low N [82]. For instance, *OsAMT1.1* is expressed in both roots and shoots and has an average of a 2.1-fold increase in its expression in response to N deprivation, which enhances ammonium uptake and increases grain yield [83]. Expression of *OsAMT1.2* in rice roots increased 8-fold due to N deficiency [82]. Similarly, in Arabidopsis, *AtAMT1.1* expression increased approximately 4-fold in response to low N supply [84]. In contrast, the expression of *OsAMT1.3* was downregulated in rice roots and produced low grain yields [82]. Hence, the regulation of these transporter genes is strongly correlated with changes in N uptake activity in roots and provides solid evidence for improving NUE in barley.

Many studies suggest that manipulation of genes from primary and secondary N assimilatory pathways is effective for improving NUE [85,86]. For instance, overexpression of glutamine synthetase (GS1) is responsible for primary N assimilation, increased grain yield in rice, wheat and maize [68,87,88]. In maize, knockout of *Gln1-3 and Gln1-4* encoding the GS1 enzyme reduced grain yield in *gln1-3* and *gln1-4* mutants, whereas its overexpression increased yield by increasing kernel number and size [87]. *TaGS2-2Ab* transgenic lines increased grain yield by 5.4%–11.1% and 8%–13.5% under low N in two consecutive years in wheat. They had longer primary roots and a higher lateral root number than the wild type, which implies high N uptake [89]. Thus, further studies would be helpful to verify these genes as good candidates for improving yield under N deficiency. Correspondingly, glutamate synthase (GOGAT) serves as a potential target for improving NUE. There are two isoforms of GOGAT—the NADH-dependent cytosolic isoform (I^{ry} N assimilation) and ferredoxin-dependent plastidic isoform (II^{ry} N assimilation) [85]. Overexpression of NADH-GOGAT in rice increased spikelet weight and panicle number per plant [90,91]. Fd-GOGAT encoded by *ABC1* gene in rice is equally important in N assimilation and carbon/nitrogen balance [92].

Amino acid biosynthesis genes, such as alanine aminotransferase incorporated from barley (*HvAlaAT*) to rice, increased biomass and grain yield under low N supply [14,93]. Accordingly, yield increased by ~30% in several transgenic rice genotypes tested under ≤50% limited N supply in field conditions [93]. Similarly, metabolite enzyme gene *Me1* derived from barley is responsible for NUtE when expressed in wheat [94], suggesting that barley is a good genetic resource for NUE improvement. Overexpression of *TaNAC2-5A* in wheat increased the tiller and spike number, grain N accumulation, thousand-grain weight under low N compared to high N with ~10% yield increment than the wild type. It also upregulated both the expression of nitrate transporters and assimilation genes [95]. Furthermore, the *ARE1* gene in rice is a strong candidate for enhancing NUE. *ARE1* mutations delayed senescence and prolonged photosynthesis, which consequently enhanced NUE [16]. When compared with wild-type rice plants, these mutants had a high root to shoot ratio and chlorophyll levels under low N supply [16]. NUE is also indirectly affected by carbon metabolism. Genes involved in N metabolism and nitrate signalling are partially regulated by sugar signalling [86,92]. For instance, overexpression of sugar transporter *AtSTP 13* improved N consumption in Arabidopsis [86]. However, further studies should be conducted to better understand the crosstalk of these genes.

7. Candidate Genes for NUE in Barley

The molecular mechanisms and functional characteristics of the genes responsible for NUE in barley have not been determined in detail. However, previous NUE research on cereal crops including rice, wheat, sorghum, maize and the model plant Arabidopsis, has shed some light on the candidate genes in barley through homologous alignment against the reference genome (Table 3). In addition, genes co-localized with QTLs identified in barley (Table 2) may be highly confident for NUE. Of these, nitrate and ammonium transporters, associated partner proteins (NAR2 families), signalling genes, amino acid biosynthesis genes, N assimilation genes and transcriptional factors play key roles in N uptake, transport, assimilation and grain filling [48,65]. Generally, low-affinity transporters (NRT1) are activated at high NO_3^- levels [96] but in barley, they can be expressed without prior exposure to NO_3^- and their activity decreases with N accumulation [97]. Recently, the *HvNRT2* gene family in barley that encodes high-affinity NO_3^- transporters were also identified as NUE candidates [19].

A total of 95 candidate genes with potential for NUE improvement across seven chromosomes in the barley genome have been mapped (Table 3; Figure 1): 12 on chromosome 1H, 16 genes each on 2H and 3H, 11 genes on 4H, 13 genes on 5H, 12 on 6H and 15 genes on 7H. They belong to several gene families, viz. ammonium and nitrate transporters, signalling genes, amino acid biosynthesis genes, N assimilation and transcriptional factors. Some gene families, such as nitrate transporters, have been reported for efficient N uptake [48]. The genes are expressed mostly in roots from seedlings (ROO1), roots after 28-day-old plants (ROO2), shoots from seedlings (LEA), senescing leaves (SEN), 4-day-old embryos (EMB), developing tillers on 3rd internode (NOD), etiolated seedlings, dark condition (ETI) and epidermal strips (EPI). Thorough identification of these candidate genes and their expression profile may enable further genetic manipulation for barley NUE improvement.

Table 3. Chromosome position of the homologous candidate genes controlling NUE in barley from Arabidopsis, rice and wheat.

Gene	Origin	Homolog in Barley	Chr	Start	End	Annotation
AtNRT1.1	Arabidopsis	*HORVU7Hr1G071600*	7H	395441113	395447440	Protein NRT1/ PTR FAMILY
AtAMT1;1, AtAMT1;3	Arabidopsis	*HORVU6Hr1G057870*	6H	377828979	377831011	Ammonium Transporter 1
AtAMT2	Arabidopsis	*HORVU3Hr1G082610*	3H	599755994	599757436	Ammonium Transporter 2
AtSTP13	Arabidopsis	*HORVU4Hr1G067450*	4H	559754962	559760152	Sugar Transporter Protein 7
AtNF-YB1-2	Arabidopsis	*HORVU1Hr1G071620*	1H	494246150	494250406	Nuclear Transcription Factor Y Subunit B
AtAMT1;3	Arabidopsis	*HORVU3Hr1G065320*	3H	497824332	497833404	ABC Transporter B Family Member 4
OsDEP1	Rice	*HORVU3Hr1G051800*	3H	375950781	375954891	Grain Length Protein
OsRGA1	Rice	*HORVU7Hr1G008720*	7H	11332739	11337421	Guanine Nucleotide-Binding Protein Alpha-1 Subunit
OsSAPK1	Rice	*HORVU2Hr1G110230*	2H	719150904	719161174	Protein Kinase Superfamily Protein
OsSAPK2	Rice	*HORVU2Hr1G029900*	2H	108667788	108672779	Protein Kinase Superfamily Protein
OsSAPK3	Rice	*HORVU5Hr1G097630*	5H	605102179	605108556	Protein Kinase Superfamily Protein
OsSAPK4	Rice	*HORVU3Hr1G082690*	3H	600013901	600018673	Protein Kinase Superfamily Protein
OsSAPK5, OsPAK7	Rice	*HORVU2Hr1G075470*	2H	543955705	543960490	Protein Kinase Superfamily Protein
OsSAPK6	Rice	*HORVU1Hr1G055340*	1H	405714931	405718538	Protein Kinase Superfamily Protein
OsSAPK8	Rice	*HORVU4Hr1G013540*	4H	47804453	47807197	Protein Kinase Superfamily Protein
OsEND93-1 *, *OsEND93-3*	Rice	*HORVU7Hr1G020850*	7H	28237803	28241820	Early Nodulin-Related
OsEND93-2	Rice	*HORVU7Hr1G020760*	7H	28084520	28085738	Early Nodulin-Related
OsAlaAT10-1, OsAlaAT4	Rice	*HORVU1Hr1G018540*	1H	68365069	68370382	Alanine Aminotransferase 2
OsAlaAT10-2	Rice	*HORVU5Hr1G014730*	5H	54487548	54492982	Alanine Aminotransferase 2
OsAlaAT3-1	Rice	*HORVU2Hr1G063740*	2H	431241063	431250440	Alanine Aminotransferase 2
OsAlaAT3-2	Rice	*HORVU2Hr1G030820*	2H	114313381	114319007	Alanine Aminotransferase 2
OsGGT1, OsGGT3	Rice	*HORVU1Hr1G070220*	1H	488758496	488762295	Alanine:Glyoxylate Aminotransferase 3
OsGGT2	Rice	*HORVU4Hr1G075360*	4H	598065082	598068656	Alanine:Glyoxylate Aminotransferase 2
OsASNase1	Rice	*HORVU2Hr1G097890*	2H	681044647	681050401	N(4)-(Beta-N-acetylglucosaminyl)-L-Asparaginase
OsASNase2	Rice	*HORVU2Hr1G123070*	2H	754633334	754644513	Isoaspartyl Peptidase/L-Asparaginase
OsASP2	Rice	*HORVU7Hr1G089290*	7H	541956174	541961050	Aspartate Aminotransferase 1
OsASP3	Rice	*HORVU6Hr1G003470*	6H	7898534	7902987	Aspartate Aminotransferase 1
OsASP4	Rice	*HORVU3Hr1G073220*	3H	552738455	552750250	Aspartate Aminotransferase 3
OsASP5	Rice	*HORVU1Hr1G074590*	1H	508562566	508569749	Aspartate Aminotransferase
OsASP6	Rice	*HORVU1Hr1G042490*	1H	308288850	308292215	Aspartate Aminotransferase
OsAS	Rice	*HORVU5Hr1G020510*	5H	94913807	94917732	Transcription Initiation Factor TFIID Subunit 8
OsGDH2-3	Rice	*HORVU2Hr1G093020*	2H	656410957	656417166	Undescribed Protein
OsGDH4	Rice	*HORVU3Hr1G048870*	3H	339064181	339071356	Glutamate Dehydrogenase
OsGS3	Rice	*HORVU4Hr1G007610*	4H	20172875	20175861	Glutamine Synthetase 1.3
OsGS4	Rice	*HORVU2Hr1G111300*	2H	722462607	722470196	Bifunctional Lysine-Specific Demethylase and histidyl-hydroxylase NO66
OsGOGAT1, OsGOGAT3	Rice	*HORVU3Hr1G063050*	3H	482165392	482176766	Glutamate Synthase 2
OsGOGAT2	Rice	*HORVU2Hr1G022920*	2H	67503162	67520099	Glutamate Synthase 1

Table 3. *Cont.*

Gene	Origin	Homolog in Barley	Chr	Start	End	Annotation
OsGOX2-3	Rice	*HORVU2Hr1G103180*	2H	699321923	699325619	L-Lactate Dehydrogenase
OsGOX4	Rice	*HORVU2Hr1G060010*	2H	399434162	399565758	L-Lactate Dehydrogenase
OsGOX5	Rice	*HORVU2Hr1G030930*	2H	115538448	115548113	L-Lactate Dehydrogenase
OsNR1, OsNR3-4	Rice	*HORVU6Hr1G003300*	6H	7696549	7701423	Nitrate Reductase 1
OsNR2	Rice	*HORVU6Hr1G079700*	6H	538505303	538508978	Nitrate Reductase 1
OsNiR1-3	Rice	*HORVU6Hr1G080750*	6H	542690954	542694406	Sulfite Reductase
OsDOF1	Rice	*HORVU7Hr1G043250*	7H	130101918	130103443	DOF Zinc Finger Protein 1
OsDOF2	Rice	*HORVU4Hr1G013890*	4H	49843958	49845261	DOF Zinc Finger Protein 1
OsDOF3	Rice	*HORVU5Hr1G097620*	5H	605046251	605048334	DOF Zinc Finger Protein 1
OsDOF4	Rice	*HORVU6Hr1G069190*	6H	479031099	479167490	Monodehydroascorbate Reductase 4
OsDOF5	Rice	*HORVU1Hr1G005390*	1H	11688712	11691059	DOF Zinc Finger Protein 1
OsNF-YB2.1-2.2	Rice	*HORVU3Hr1G087390*	3H	621114774	621118012	Nuclear Transcription Factor Y Subunit B
OsNF-YB2.3	Rice	*HORVU7Hr1G105460*	7H	617016382	617017035	Nuclear Transcription Factor Y Subunit B-2
OsHLHm1	Rice	*HORVU4Hr1G065640*	4H	547060963	547062633	Basic Helix-Loop-Helix (bHLH) DNA-Binding Superfamily Protein
OsHLHm2	Rice	*HORVU4Hr1G009440*	4H	26788350	26791410	Basic Helix-Loop-Helix (bHLH) DNA-Binding Superfamily Protein
OsHLHm3	Rice	*HORVU3Hr1G079340*	3H	583076029	583165960	Leucine-Rich Repeat Protein Kinase Family Protein
OsHLHm4	Rice	*HORVU5Hr1G002090*	5H	6036768	6041581	Basic Helix-Loop-Helix (bHLH) DNA-Binding Superfamily Protein
OsNAC006	Rice	*HORVU4Hr1G012030*	4H	38610964	38613054	NAC Domain Protein
OsNAC6	Rice	*HORVU7Hr1G106480*	7H	619955492	619960319	NAC Domain Containing Protein 1
OsNAC9/OsSNAC1	Rice	*HORVU5Hr1G111590*	5H	636772198	636774461	NAC Domain Protein
OsNAC10	Rice	*HORVU5Hr1G045650*	5H	353125420	353127305	NAC Domain Protein
OsAPO1/OsFBX202	Rice	*HORVU7Hr1G108970*	7H	626595594	626597285	Aberrant Panicle Organization 1 Protein
OsFBX94	Rice	*HORVU5Hr1G025530*	5H	140302431	140306350	F-Box Only Protein 13
OsNRT2.3a-2.3b	Rice	*HORVU3Hr1G066090*	3H	503310428	503312717	High-Affinity Nitrate Transporter 2.6
OsNAR2.1-2.2	Rice	*HORVU5Hr1G115500*	5H	646682607	646686179	High-Affinity Nitrate Transporter 3.1
OsLHT1	Rice	*HORVU7Hr1G032060*	7H	65594488	65596772	Lysine Histidine Transporter 2
OsLHT2	Rice	*HORVU7Hr1G074660*	7H	428023559	428028502	Transmembrane Amino Acid Transporter Family Protein
OsCKX2/Gn1a	Rice	*HORVU3Hr1G027430*	3H	116879865	16883601	Cytokinin Dehydrogenase 2
OsCKX5	Rice	*HORVU3Hr1G075920*	3H	567046659	567052020	Cytokinin Dehydrogenase 5
OsCKX4	Rice	*HORVU3Hr1G105360*	3H	668168109	668176192	Cytokinin Oxidase/Dehydrogenase 1
OsCKX3	Rice	*HORVU1Hr1G042360*	1H	306444595	306450221	Cytokinin Dehydrogenase 3
OsCKX1	Rice	*HORVU3Hr1G019850*	3H	58407698	58410314	Cytokinin Oxidase/Dehydrogenase 6
OsCKX7	Rice	*HORVU7Hr1G086710*	7H	522868134	522870101	Cytokinin Dehydrogenase 10
OsCKX8	Rice	*HORVU1Hr1G057860*	1H	421966219	421973332	Cytokinin Oxidase/Dehydrogenase 1
OsCKX9	Rice	*HORVU6Hr1G039680*	6H	207624575	207626177	Cytokinin Oxidase/Dehydrogenase 1
OsIPT1-2	Rice	*HORVU1Hr1G011480*	1H	27827675	27830691	tRNA Dimethylallyltransferase

Table 3. *Cont.*

Gene	Origin	Homolog in Barley	Chr	Start	End	Annotation
OsIPT3	Rice	*HORVU3Hr1G025950*	3H	103350630	103351969	tRNA Dimethylallyltransferase
OsIPT4-5	Rice	*HORVU5Hr1G110100*	5H	631892524	631893928	tRNA Dimethylallyltransferase 2
OsCIN1-2	Rice	*HORVU4Hr1G086300*	4H	633598303	633602296	Beta-Fructofuranosidase, Insoluble Isoenzyme 1
OsCIN3	Rice	*HORVU4Hr1G011000*	4H	33449700	33451633	Beta-Fructofuranosidase, Insoluble Isoenzyme 3
OsSGR1	Rice	*HORVU5Hr1G081500*	5H	564845582	564848348	Protein STAY-GREEN Chloroplastic
OsFNR1	Rice	*HORVU2Hr1G038830*	2H	184566812	184570474	Ferredoxin–NADP Reductase
OsFNR2	Rice	*HORVU5Hr1G103180*	5H	615129595	615133117	Ferredoxin–NADP Reductase
OsARE1	Rice	*HORVU7Hr1G063720*	7H	314391516	314425666	Chloroplast envelope membrane protein
TaAS1-3A	Wheat	*HORVU3Hr1G013910*	3H	31212143	31216892	Asparagine synthetase [glutamine-hydrolyzing]
TaASN2-1A	Wheat	*HORVU1Hr1G084370*	1H	533821309	533827604	Asparagine synthetase [glutamine-hydrolyzing] 2
TaASN2-1B	Wheat	*HORVU1Hr1G092110*	1H	549769608	549775894	Asparagine synthetase [glutamine-hydrolyzing] 2
TaANR1-6A	Wheat	*HORVU6Hr1G073040*	6H	507069039	507080622	MADS-box transcription factor 57
TaGS1.1-4A	Wheat	*HORVU4Hr1G066860*	4H	555801831	555805679	Glutamine synthetase 1
TaGDH1-5A	Wheat	*HORVU5Hr1G104700*	5H	619890137	619895338	Glutamate dehydrogenase 1
TaNRT2.1, TaNRT2.4-6A	Wheat	*HORVU6Hr1G005600*	6H	12385615	12387964	High-affinity nitrate transporter 2.6
TraesCS6B01G041800	Wheat	*HORVU7Hr1G120020*	7H	650777327	650785628	Disease resistance protein
TraesCS6B01G043500	Wheat	*HORVU6Hr1G005690*	6H	12565857	12569544	Disease resistance protein
TraesCS6B01G051000	Wheat	*HORVU3Hr1G098450*	3H	658650524	658656351	Receptor kinase 3
TraesCS2A01G128200	Wheat	*HORVU0Hr1G002520*	Un	11160951	11162387	UDP-Glycosyltransferase
TraesCS2A01G127800	Wheat	*HORVU2Hr1G124210*	2H	757856039	758101641	Glutathione-regulated
TraesCS2A01G128400	Wheat	*HORVU2Hr1G022450*	2H	65225047	65230215	Chromodomain-helicase-DNA-binding
TraesCS6B01G194500	Wheat	*HORVU6Hr1G033850*	6H	156256740	156263950	Chaperone protein DnaJ
TraesCS2A01G130100LC	Wheat	*HORVU7Hr1G102500*	7H	611628889	611629721	Phosphoinositide phospholipase C
TraesCS6B01G050700	Wheat	*HORVU6Hr1G006880*	6H	14328001	14332255	Carboxypeptidase Y homolog A

This list of candidate genes is based on several recent reviews from which the homologous genes in barley were identified [48,60,61,78,98,99]. The gene sequences of rice and wheat which were BLAST-searched against barley can be downloaded from http://rice.plantbiology.msu.edu/analyses_search_locus.shtml) and https://plants.ensembl.org/Triticum_aestivum/Info/Index, respectively. Gene IDs and their positions on the barley reference genome and other relevant information are available from IPK Barley BLAST Server and Ensembl Plants using default BLAST parameter settings (https://apex.ipkgatersleben.de/apex/f?p=284:10, http://webblast.ipkgatersleben.de/barley_ibsc/, https://plants.ensembl.org/Hordeum_vulgare/Tools/Blast?db=core).

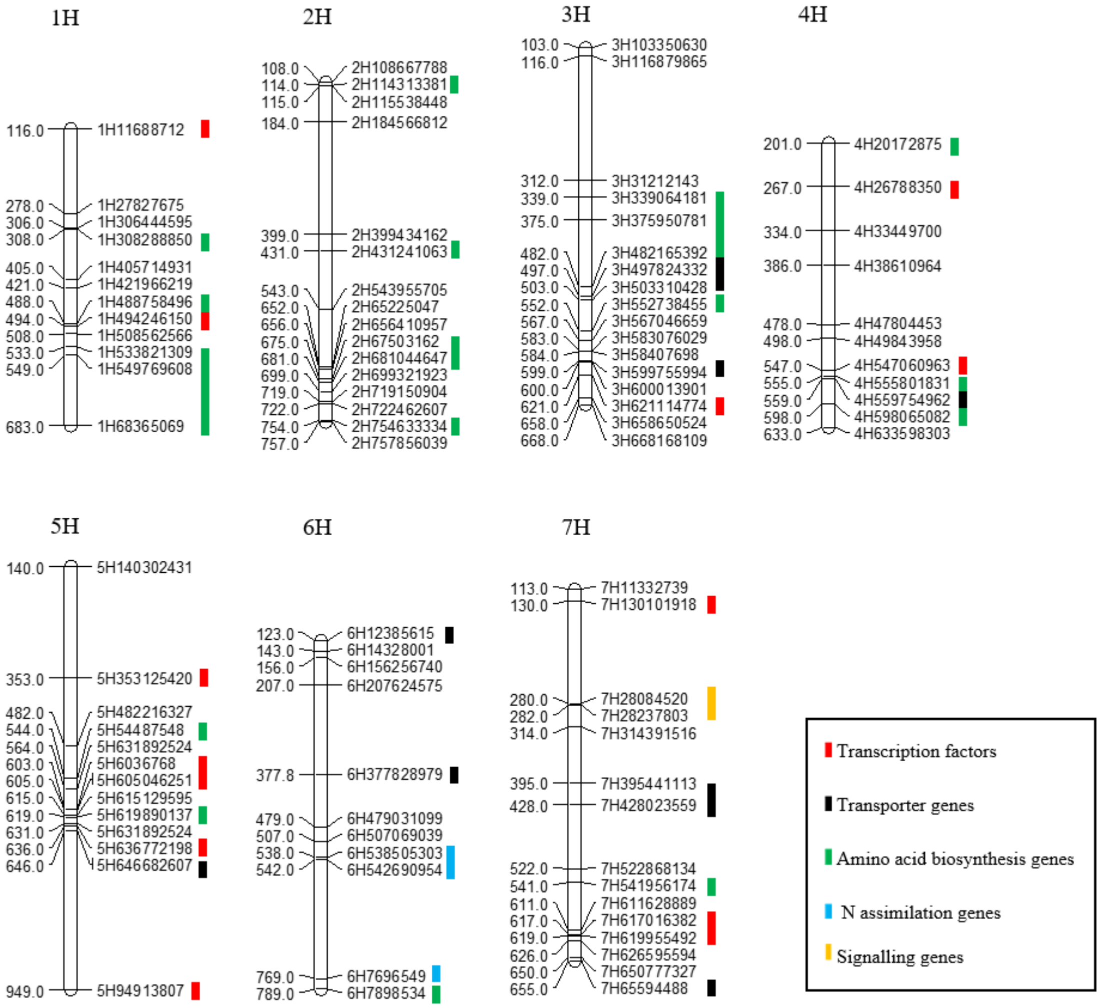

Figure 1. Physical positions of the major candidate gene families for NUE on barley chromosomes. The candidate gene families based on their annotation are indicated on the right side of the chromosome (MapChart 2.32: https://www.wur.nl/en/show/Mapchart.htm).

8. CRISPR/Cas9 Genome Editing for Barley NUE Improvement

Conventional plant breeding is categorized mainly as classical and molecular breeding [100,101]. Classical breeding involves parental crossing to produce improved cultivars by phenotypic analysis over generations. Molecular breeding extends to marker-assisted selection (MAS) and genetic modifications. The newly emerging genome-editing technologies that are correlated with the precise manipulation of an organism's DNA by the alteration, insertion or deletion of targeted locations in the genome hold a prominent place in plant genomic research. Several approaches have evolved from HR-mediated targeting—from cre-lox editing, zinc finger nucleases (ZFNs) and transcription-like effector nucleases (TALENs) to the most commonly used clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 (CRISPR/Cas9) genome editing [102–106]. Compared with ZFNs and TALENs that need expertise in protein engineering, the CRISPR/Cas system needs only two components—Cas9 endonuclease and guide RNA (sgRNA)—which comprise CRISPR RNA and trans-activating CRISPR RNA (crRNA-trcrRNA) transcript. The sgRNA guides the Cas-9 protein, which causes double-strand breaks, to the target site [107]. The CRISPR/Cas system also facilitates multiplex genome editing, high-efficiency targeting and easy customization [105] and is thus more precise, accurate and cost-effective than previous technologies.

The CRISPR/Cas9 system was first used in 2013 in rice and wheat targeting four rice genes and one wheat gene [108]. Recent studies have applied the technology in cereal crops, including wheat, rice, maize, barley and sorghum, to genetically improve yields or nutrient values or to overcome harsh environmental conditions, such as biotic and abiotic stresses [109–112]. CRISPR/Cas9 was successfully used to target *ZmIPK* gene in maize to reduce phytic acid contents in maize, and further increase mineral nutrient value [113]. It has also generated new variants of *ARGOS8* gene in maize to increase yields under drought stress [114]. Disease resistance in crop plants is another major aspect of CRISPR/Cas9 application, e.g., the development of rice mutant lines to resist blast fungal pathogen by targeting *OsERF922* gene [115], wheat mutant lines to induce powdery mildew resistance by targeting *TaMLO-A1, TaMLO-B* and *TaMLO-D* genes [111], and a non-transgenic cucumber line, resistant to cucumber vein yellowing disease, papaya ringspot mosaic virus-W and zucchini yellow mosaic virus [116]. In addition, CRISPR/Cas9 was carried out to mutate *OsHKT1;4* in rice to study its nutrient use efficiency [117]. CRISPR/Cas9 genome editing was recently used in barley for the first time, targeting *HvPM19* to identify its potential for mutation induction and stable transmission, and generated transgene-free plants with the desired mutation [109]. This recent study on barley and other successful applications of CRISPR/Cas9 genome editing are proof for the potential improvement in NUE in barley. To date, most of the genetic studies focussed on overexpression of the genes to improve NUE [95,118]. Hence, the use of CRISPR/Cas9 to downregulate or knockdown genes would be a better approach to improve NUE in barley. For instance, the homolog of rice *ARE1* gene [16], which is a promising locus for NUE improvement, might be downregulated to improve nitrogen use efficiency in barley.

9. Conclusions and Perspectives

Excessive use of N fertilizers in crops to boost grain yields is a major cause of soil, water, and air pollution and greenhouse gas emissions. It also has a worldwide economic impact due to the high production costs of N fertilizer. Hence, improving NUE is very important for environmentally friendly, profitable crop production. Genetic improvement of NUE should be a priority to address this issue, although proper management of N fertilizer through agronomic practices is possible. NUE is a polygenic trait that is difficult to quantify. To date, no direct selection criteria have been available for high NUE genotypes other than some agronomic traits, such as root and shoot dry biomass, for conventional breeding.

N fertilization affects the protein content in barley, which is a major concern. Only limited research has been conducted on barley NUE. A few QTLs controlling NUE have been identified, but they are not stable across experiments due to low marker density, limited genetic diversity and small population size. Thus, incorporation of knowledge from other crops such as rice, maize and wheat is desirable to generate a candidate gene pool for NUE improvement. Homologs of these genes can be blast-searched against the genome sequence of barley, and further experiments can be designed to understand the molecular mechanisms of them in barley NUE improvement.

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