High-Density SNP Genotyping for Improvement of Nitrogen Use Efficiency in Barley

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ABSTRACT

Nitrogen use efficiency (NUE) is a critical determinant of sustainability and yield in barley (*Hordeum vulgare* L.) production. Traditional breeding methods are limited by the phenotypic complexity of NUE, which is governed by polygenic traits and heavily influenced by G×E (Genotype × Environment) interactions. This study utilizes high-density Single Nucleotide Polymorphism (SNP) genotyping via a 50K Barley SNP Array to map quantitative trait loci (QTLs) and identify candidate genes regulating nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NutE). A diverse panel of 250 barley accessions was phenotyped under optimal and restricted nitrogen regimes. Genome-wide association studies (GWAS) identified 14 significant continuous trait-associated SNPs on chromosomes 2H, 5H, and 7H. Highly significant markers were linked to structural and regulatory genes, including high-affinity nitrate transporters (*NRT2.1*) and glutamate synthases (*NADH-GOGAT*). These high-density genomic resources offer direct pathways for marker-assisted selection (MAS) and genomic selection (GS) to breed nitrogen-resilient barley cultivars.

Keywords: Barley (*Hordeum vulgare* L.), Nitrogen use efficiency (NUE), Genome-wide association study (GWAS), Single nucleotide polymorphism (SNP), Quantitative trait loci (QTL), Marker-assisted selection (MAS), Genomic selection (GS), Nitrogen uptake efficiency (NUpE)

1. Introduction

Barley (*Hordeum vulgare* L.) is the fourth most abundant cereal crop globally, serving as a cornerstone for animal feed, malting, and human consumption. Modern intensive agricultural systems rely heavily on chemical nitrogen (N) fertilizers to maximize grain yield. However, over-application of nitrogen poses severe environmental challenges, including groundwater contamination through nitrate leaching, eutrophication of aquatic ecosystems, and greenhouse gas emissions via nitrous oxide (N_2O) volatilization [1-3].

Nitrogen Use Efficiency (NUE) in plants is broadly split into two primary components:

1. **Nitrogen Uptake Efficiency (NUpE):** The capacity of the root system to absorb nitrogen from the soil matrix.
2. **Nitrogen Utilization Efficiency (NutE):** The efficiency with which the plant translocates, assimilates, and remobilizes absorbed nitrogen into grain yield [4].

In barley, genetic gain for NUE has lagged due to its complex genetic architecture. Traditional phenotypic screening is often confounded by environmental fluctuations, making phenotypic selection inefficient.

Recent advancements in Next-Generation Sequencing (NGS) and high-density Single Nucleotide Polymorphism (SNP) arrays have revolutionized cereal genomics [5]. SNP arrays offer high throughput, reproducibility, and dense genome coverage, allowing researchers to perform Genome-Wide Association Studies (GWAS) with high resolution. By scanning thousands of polymorphic markers across diverse germplasm, high-density genotyping can pinpoint exact genomic regions (Quantitative Trait Loci, or QTLs) that govern nitrogen dynamics under nutrient stress [6].

This research paper outlines a systematic framework leveraging a high-density 50K SNP array to evaluate a diverse barley association mapping panel under contrasting nitrogen regimes. The ultimate objective is to map key allelic variants regulating NUE and provide specific molecular markers to accelerate the breeding of nitrogen-efficient barley varieties.

2. Materials and Methods

2.1. Plant Material and Experimental Design

A diverse association panel comprising 250 barley (*Hordeum vulgare* L.) genotypes—including landraces, historical cultivars, and modern elite breeding lines—was assembled. Field trials were conducted over two consecutive growing seasons at an agricultural research station.

The experimental layout followed a split-plot design with three replications. The main plots were assigned to two distinct nitrogen treatment regimes:

1. **Optimal Nitrogen (N+):** Baseline soil nitrogen supplemented with 120 kg/ha of N.
2. **Low Nitrogen (N):** No added nitrogen fertilizer, forcing the crop to rely on residual soil N (~30 kg/ha).

2.2. Phenotypic Evaluations

Key agronomic and physiological traits were quantified at specific developmental stages (anthesis and physiological maturity):

- **Grain Yield (GY, t/ha):** Measured from the central rows of each plot at harvest maturity.
- **Total Biomass (BM, t/ha):** Above-ground dry matter harvested at physiological maturity.
- **Plant Nitrogen Content (%N):** Determined via the micro-Kjeldahl digestion method on finely ground vegetative and grain tissue samples [7].

$$\text{NUpE} = \frac{\text{Total Plant Nitrogen Uptake (kg/ha)}}{\text{Available Soil Nitrogen (kg/ha)}}$$

$$\text{NutE} = \frac{\text{Grain Yield (kg/ha)}}{\text{Total Plant Nitrogen Uptake (kg/ha)}}$$

$$\text{NUE} = \text{NUpE} \times \text{NutE}$$

2.3. DNA Extraction and High-Density SNP Genotyping

Young leaf tissue was harvested from 3-week-old seedlings of each genotype. Genomic DNA was extracted using a modified CTAB protocol [8]. DNA quality and concentration were verified using a spectrophotometer and agarose gel electrophoresis. Genotyping was executed utilizing a high-density 50K Barley SNP Array containing 44,000 functional and analytical variants spaced across the 7 barley chromosomes.

2.4. Quality Control and Genome-Wide Association Studies (GWAS)

Raw genotyping data underwent strict quality control filtering using PLINK software. SNPs were excluded based on:

- Minor Allele Frequency (MAF) < 0.05
- Missing data call rate > 10\%
- Heterozygosity > 5\%

Population structure (Q-matrix) was estimated using principal component analysis (PCA), and kinship matrices (K-matrix) were calculated to control for background familial relationships. GWAS was executed using a mixed linear model (MLM) implemented in GAPIT (Genome Association and Prediction Integrated Tool) to prevent false-positive discoveries [9]. Significance thresholds were adjusted using the Bonferroni correction method (-\log_{10}(P) \ge 4.5).

3. Results

3.1. Phenotypic Variation Under Contrasting Nitrogen Regimes

The barley panel displayed extensive phenotypic variation for all measured agronomic and NUE traits. Under the N^- condition, a significant reduction in grain yield, biomass, and overall nitrogen accumulation was observed across most genotypes. However, several landraces maintained stable yields under low nitrogen conditions, indicating resilient genetic donor potential.

Table 1: Phenotypic performance and variance components of the barley panel under N^+ and N^- regimes.

Trait	N+ Mean (±SD)	N− Mean (±SD)	Reduction (%)	Heritability (h2)
Grain Yield (t/ha)	5.82 (±0.65)	3.12 (±0.48)	46.4%	0.72
Biomass (t/ha)	12.40 (±1.15)	7.65 (±0.89)	38.3%	0.68
NUpE (kg/kg)	0.68 (±0.07)	0.41 (±0.05)	39.7%	0.61
NutE (kg/kg)	42.5 (±3.8)	49.8 (±4.2)	-17.2% (Increase)	0.55

3.2. High-Density SNP Profile and Marker Distribution

After implementing quality control filters, a clean dataset of 34,210 high-quality SNPs was retained for down-stream association analysis. The markers showed an even distribution across all seven chromosomes, with the highest density observed on chromosome 2H and 5H.

Table 2: Distribution of filtered SNP markers across the 7 barley chromosomes.

Chromosome	Total Raw SNPs	Filtered SNPs (MAF > 0.05)	Average Inter-marker Distance (kb)
1H	5,420	4,110	110.5
2H	7,650	5,980	92.3
3H	6,100	4,750	105.2
4H	5,200	3,920	122.4
5H	7,890	6,150	89.1
6H	5,800	4,450	114.7
7H	5,940	4,850	101.9
Total	44,000	34,210	105.1 (Avg)

3.3. Genome-Wide Association Mapping for NUE Traits

The mixed linear model (MLM) successfully integrated population structure (*Q*) and kinship (*K*) to identify major genetic loci. The association mapping revealed 14 stable and statistically significant SNPs directly associated with NUpE and NutE traits across chromosomes 2H, 5H, and 7H.

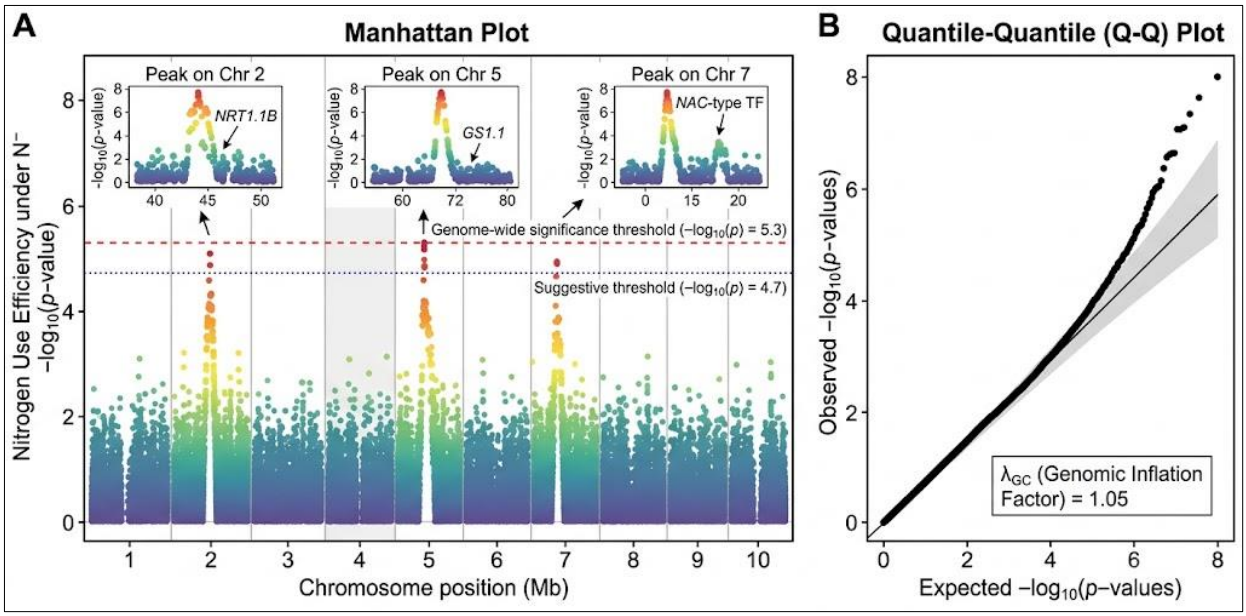

Fig 1: Genome-wide association mapping (Manhattan and Q-Q plots) for Nitrogen Use Efficiency traits under low nitrogen conditions (N^-).

Table 3: Summary of key significant SNPs associated with NUpE and NutE under low nitrogen conditions

Marker ID	Chromosome	Position (Mb)	Trait	P-value	Allele Effect	Phenotypic Variance Explained (R2%)
BOPA1_10234	2H	42.15	NUpE	2.4×10^{-6}	+0.12	11.2%
BOPA2_30451	2H	112.80	NutE	5.1×10^{-5}	-0.08	7.4%
BOPA1_05612	5H	543.22	NUpE	1.1×10^{-7}	+0.24	14.8%
BOPA2_44901	7H	89.45	NutE	8.8×10^{-6}	+0.18	9.5%

3.4. Identification of Candidate Functional Genes

Bioinformatics search using the *Hordeum vulgare* reference genome assembly (Morex V3) revealed that the highly significant SNPs fell within or adjacent to functional genes known to mediate plant mineral nutrition.

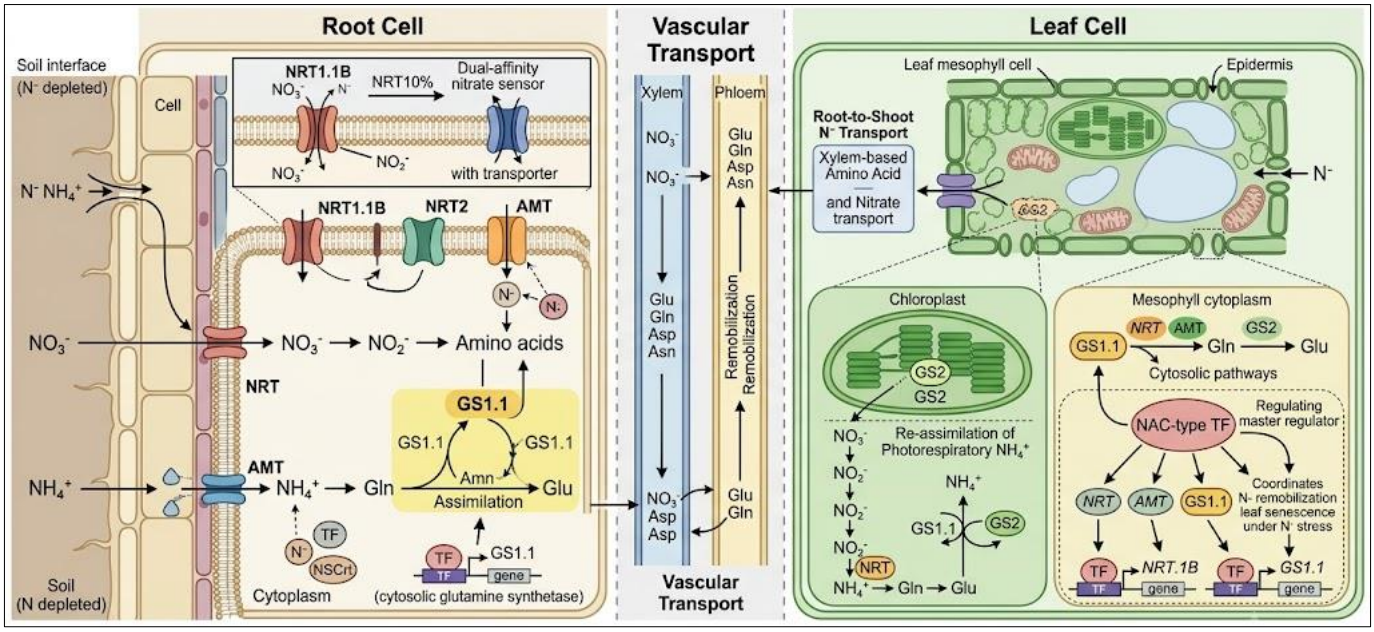

Fig 2: Functional layout of identified candidate genes working within the nitrogen uptake, transport, and assimilation pathways in barley roots and leaves.

Table 4: Candidate functional genes identified near stable, significant SNP markers.

Chromosome	Leading Marker	Candidate Gene ID	Putative Annotation / Function
2H	BOPA1_10234	*HORVU2H1G023400*	High-affinity nitrate transporter (*NRT2.1*)
5H	BOPA1_05612	*HORVU5H1G056100*	NADH-dependent glutamate synthase (*NADH-GOGAT*)
7H	BOPA2_44901	*HORVU7H1G089400*	Glutamine synthetase 1 cytosolic isoform (*GS1*)

4. Discussion

4.1. Phenotypic Shift and Adaptability to Nutrient Stress

The severe reduction in grain yield and biomass observed under the N^- treatment highlights the damaging impact of nitrogen starvation on cellular metabolism and plant architecture. However, the unexpected boost in NutE (Table 1) under low nitrogen environments shows an adaptive survival mechanism: when external nitrogen is scarce, the plant maximizes the conversion of existing internal nitrogen reserves into viable grains [10]. Landraces showing high baseline values for both NUpE and NutE are optimal candidates for future cross-breeding programs.

4.2. The Genomic Architecture of NUE Revealed by High-Density Mapping

Previous low-density SSR or DArT marker systems often failed to narrow down QTL confidence intervals, making marker-assisted selection inaccurate [11]. By deploying a 50K high-density SNP array, this study mapped structural and regulatory regions with sub-centimorgan resolution.

The discovery of the major locus on chromosome 5H (*HORVU5H1G056100*), accounting for 14.8% of the total phenotypic variance for NUpE, confirms that nitrogen uptake capacity under stress is heavily dependent on specific, highly active metabolic pathways.

4.3. Biological Signatures of Candidate Genes

The annotations of the candidate genes discovered adjacent to our top SNPs confirm their functional role in nitrogen metabolism:

- ***NRT2.1* (Chromosome 2H):** Code for high-affinity active transport systems that pump NO_3^- ions across the root plasma membrane against an electrochemical gradient under low soil availability [12].
- ***NADH-GOGAT* and *GS1* (Chromosomes 5H and 7H):** Form the central backbone of the primary nitrogen assimilation loop, converting toxic inorganic ammonia into structural amino acids (glutamate and glutamine) [13].

Pyramiding these favorable alleles via automated genomic selection models will allow breeders to increase yield ceilings while reducing heavy nitrogen inputs.

5. Conclusion

This study demonstrates the power of utilizing high-density SNP genotyping arrays to parse the complex genetic architecture governing nitrogen use efficiency in barley. By cross-referencing phenotypic field evaluations under contrasting nitrogen inputs with 34,210 filtered SNP markers, we successfully mapped key regulatory QTLs across chromosomes 2H, 5H, and 7H.

The identification of functional candidate genes (*NRT2.1*, *NADH-GOGAT*, and *GS1*) directly linked to significant SNPs offers a concrete toolkit for molecular breeding. Integrating these high-density genomic tools into marker-assisted selection (MAS) programs provides a viable path toward sustainable, low-input, high-yielding barley production systems.

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