

Review



Maximizing nitrogen stress tolerance through high-throughput phenotyping in rice[☆]

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ARTICLE INFO

Keywords:

High throughput phenotyping
Phenomics
Rice (*Oryza sativa* L.)
Nitrogen use efficiency (NUE)
Nitrogen
Stress tolerance

ABSTRACT

Nitrogen (N) is a significant nutrient element limiting rice yield and quality, a major staple crop consumed worldwide. N deficiency negatively affects the growth and development of rice by impacting vital physiological processes. Plants have developed multiple resilience strategies, including enhanced nitrogen use efficiency (NUE) to cope with N-deprived situations. NUE in rice is less than 40 %, and increased N application leads to high production costs and ecosystem damage. Improving NUE has been one of the major challenges of agriculture research in the recent past. NUE is an obfuscated trait governed by diverse physiological traits and controlled by complex genetic mechanisms. In recent years, a combination of multi-omics techniques (phenomics and genomics) has enhanced the N resilience maximization efforts of the agricultural research community. Phenomics technology has displayed the ability to perform systematic, organism-wide phenotyping of N stress response in diverse crops over the entire life cycle using non-invasive sensors on high throughput platforms (HTPs) in a more precise manner. These HTPs augment precision phenotyping (at the spatiotemporal scale) of component traits of NUE, which are difficult to phenotype mainly due to its dynamic interactive nature with the environment. Phenomics has drastically reduced the phenotype-genotype gap by optimally utilising other omics data for breeding climate smart cultivars with enhanced N stress tolerance. This review focuses on the recent advances in HTP-based phenotyping of NUE-related traits to identify novel QTLs/genes/signaling pathways associated with improved NUE both in controlled environments and field conditions.

1. Rice – a key to food security

Rice (*Oryza sativa* L.) is a staple food, a valuable source of starch, protein and mineral nutrients for half of the global population. It plays a vital role in providing sustenance for more than half of the world's population (Lee, 2021). Over the past five decades, global rice

production has significantly increased to meet the growing food grain demands and achieve food security. Globally, the harvested area was measured as 1.65×10^8 hm², while production was found to be 7.76×10^8 tons in 2022, respectively. In India, harvested area and production measures for 2022 include 4.64×10^7 hm² and 1.96×10^8 tons, respectively (FAOSTAT, 2023). However, further increases in rice

[☆] This article is part of a special issue entitled: “Deciphering Plant Stress Signaling Networks: Implications for Resilience and Microbiome Interactions” published at the journal *Plant Stress*.

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<https://doi.org/10.1016/j.stress.2025.100764>

Received 17 October 2024; Received in revised form 3 February 2025; Accepted 5 February 2025

Available online 5 February 2025

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productivity are facing a multitude of challenges under the changing global climatic conditions. The unprecedented fluctuations and changes in environmental conditions such as abiotic stress (drought, flooding, rainfall, nutrients, etc.) have profound impacts on plant growth, development and yield which thereby leads to food security (Rezvi et al., 2023; Ramlal et al., 2023). Similarly, rice is prone to nutrient stresses including the availability of nitrogen (N) which is a major yield-limiting factor.

N is a major nutrient element vital for all crops including rice required for growth and development. In South Asian countries and the US, N is applied at 140–150 kg N ha⁻¹ while in Japan it is only about 80 kg N ha⁻¹ and some of the N is lost in gaseous form and expected to reach 18.8 kg N yr⁻¹ by 2030 (Farooq et al., 2024). However, the natural N content in the soil is often insufficient to meet the demands of intensive rice cultivation practices. As a result, the application of N fertilizers has become indispensable to enhance yield and production. In the last 50 years (1972–2022), the use of chemical-based N fertilizers has many disadvantages and has increased by three folds, with an application of 30 million tons of pure N. N stress negatively impacts plant's performance such as impairing chlorophyll content, affecting photosynthesis, growth rate, tiller number and yield. Furthermore, the excessive use of N fertilizers in rice cultivation has led to environmental problems (soil degradation, eutrophication and greenhouse gas emissions) (Baweja et al., 2020; Vishnukiran et al., 2020; Dhankhar and Kumar, 2023). Thus, improving rice yield with less fertilizer input is the top priority in developing countries to sustain food security and the ecosystem (Wing et al., 2018; Hickey et al., 2019). Several breeding strategies were formulated for developing new high-yielding genotypes with higher input use efficiency by integrating germplasm accessions, genomic resources and advanced molecular breeding technologies (Ali et al., 2018; Wing et al., 2018; Wang et al., 2020). Phenotyping is the most important aspect of any crop improvement strategy. Tuberosa (2012) stated that “phenotyping is king, and heritability is queen” in the context of germplasm diversity, phenotyping method type and quality of genotypic information for crop improvement. Phenotyping for N-related traits and nitrogen use efficiency (NUE) is important due to a poor understanding of crop responses towards them (Sharma et al., 2018). Vijayalakshmi et al. (2015) and Rao et al. (2018) have described manual destructive methodologies for phenotyping NUE-related traits while others are exploring next-generation phenotyping technology for non-invasive estimation of NUE traits in diverse crops except rice (Dodig et al., 2019; Nguyen et al., 2019a,b).

Germplasm resources are fundamental resources for genetic improvement and functional genomics research to support the breeding of climate-resilient crop varieties. Rice is very advantageous in terms of possessing genetically rich diversity, wider geographical adaptability and huge genomics resources (Wang et al., 2018c; Zhao et al., 2018). Zhao et al. (2011) broadly classified the rice germplasms into five subpopulations viz., aromatic, aus, indica, temperate japonica and tropical japonica. Wang et al. (2018c) studied the diversity of 3,010 Asian cultivated rice genotypes and reported several new unreported subpopulations that correlate with geographic location apart from previously identified five major groups. Higgins et al. (2020) analyzed 672 Vietnamese rice genomes and identified a new subpopulation of Indica-5 (I5) from low-lying Mekong and Red River Deltas in Vietnam. Among the populations, diverse rice landraces containing natural variations are considered to be important genetic resources for enhancing the N stress tolerance in elite cultivars (Pinson et al., 2015; Hawkesford and Griffiths, 2019).

However, appropriate genetic resources and high-resolution genomic information are available in rice. The presence of high genetic complexity of NUE traits and low-resolution phenotyping methodologies pose a typical “phenotype and genotype gap” in improving the NUE of rice. Therefore, a complete understanding of prior art information on the genetic architecture of complex NUE traits and recent advances in next-generation phenotyping & genotyping methodologies are

very critical to strategically integrating phenomics and genomics technologies for rice crop improvement.

This review deals with recent advances in high throughput platforms (HTP)-based phenotyping of nitrogen use efficiency (NUE)-related traits to identify novel genes and pathways associated with improved NUE both in controlled environments and field conditions. It will also provide challenges and plausible solutions that occur in HTP-based utilization of technology and applications of NUE in rice breeding and crop improvement programs.

2. Nitrogen use efficiency in rice

Nitrogen is a major essential nutrient for all plants. Among the cereals, rice has the lowest NUE (~33 %) and has not significantly increased in the last few decades (Omara et al., 2019). Reduced N supply is a limiting factor for yield and grain quality of rice, yet its excessive application leads to crop lodging, increased incidence of pests and diseases, high production cost and ecosystem damage. NUE plays a crucial role in achieving the sustainable development goals and agricultural growth to reach 67 % by 2050 to meet the global food demand (Ladha et al., 2020). As the average NUE in cereals does not exceed 50 %, research on improving NUE in crops has gained enormous significance in sustainable agriculture (Kubota et al., 2018; Omara et al., 2019; Salim and Raza, 2020). In India, NUE reduced from approximately 40 % to 30 % in 50 years from 1961 to 2011 (Zhang et al., 2015). Raun and Johnson (1999) used the equation apparent N recovery which represents the portion of applied N fertilizer used by the crops.

Multiple approaches aiming at sustainable agriculture, including precision farming, application of slow-release fertilizers, use of bio-fertilizers and legume-based crop rotations, have been shown to improve NUE (Cui et al., 2018). However, improving the inherent NUE of rice through breeding N resilient genotypes is considered a sustainable strategy. Conventionally, phenotypic trait values of complex NUE traits are measured in a large population of rice genotypes and novel genetic resources are selected for cross-breeding strategy. Most of the NUE traits are derived from biomass, yield and tissue N concentration estimated at the end of the season. NUE consists of two main physiological components viz, nitrogen uptake efficiency (NUpE) and nitrogen utilization efficiency (NUE) (Xu et al., 2012). NUE can be hypothetically improved by either NUpE, NUE or both (Han et al., 2015; Hawkesford and Griffiths, 2019). The NUE is mostly polygenic and quantitative traits as it is governed by multiple processes involving nitrogen sensing, uptake, transport, assimilation, and remobilization (Fan et al., 2017; Xu and Takahashi, 2020). The important approach for developing cultivars with better NUE is related to not only enhanced yield but also higher N content in grains under low and sufficient N supply conditions. It is also imperative to compare the performances of genotypes under various N conditions to identify NUE-efficient genotypes.

NUE and its component traits, viz, uptake, utilization, content, and availability of N (Table 2). One simple definition is yield per unit of applied N. It is also mostly derived as a product of NUpE and NUE (Han et al., 2015). Conversely, NUE can be deliberated in terms of utilization index, that is, the biomass produced per unit of applied N (NUEb) (Siddiqi and Glass, 1981). Another empirical way is to estimate the grain yield per unit of available N (NUEg). However, rice often produces large biomass per unit of supplied N without producing grain yield, thus showing a low NUEg (Han et al., 2015). Therefore, protein content per unit grain weight should be considered as appropriate to estimate NUE, and it represents the efficient uptake and subsequent utilization of N. Rao et al. (2018) concentrated on physiological nitrogen use efficiency, internal efficiency and nitrogen harvest index (NHI) to identify the superior rice landraces with promising yields under N stress conditions. It is important to compare the performance of a genotype under normal N supply with that under N-deficit conditions to identify N-responsive genotypes and genomic regions important for NUE and N-deficiency stress tolerance. Toward this, a new index called the N uptake efficiency

susceptibility index has been proposed.

2.1. Nitrogen uptake

NUE is a complex trait consisting of NUpE, (NUE), partitioning (HI), trade-offs between yield and quality (grain N), and crosstalk with the capture and utilization of other nutrients (Hawkesford and Riche, 2020). NUpE is defined as the proportion of plant N uptake upon the total N available in soil and is expressed as kg N in plant per kg available N in soil (Good et al., 2004). The NUE is the efficiency of assimilation and remobilization of the plant's N for the production of grains (Xu et al., 2012). It has been observed that in rice both soil edaphic and environmental factors strongly influence the NUpE (Plett et al., 2020). Rice prefers to uptake N in the form of ammonium (NH_4^+) under puddled transplanted cultivation, where it is readily available in the soil at pH \sim 5.5. However, roots can uptake both inorganic forms (nitrate (NO_3^-) and ammonium) and organic forms (urea, amino acids and peptides). NUpE intensely depends on NO_3^- and NH_4^+ transport at the root level. N in its inorganic form (NO_3^-) is regularly remobilized between different tissues to optimize NUE during plant development, while organic N can also be remobilized. In soils, the NO_3^- is highly mobile in nature and internally moves through a mass flow mechanism, and its uptake is mediated through low-affinity nitrate transporter 1/peptide transporter families (NRT1/NPF) and high-affinity transporter (NRT2) (Plett et al., 2010; Liu et al., 2022). NH_4^+ absorption is regulated by ammonium/methyl ammonium transporters (AMTs) (Yan et al., 2011). The NH_4^+ derived from NO_3^- or absorbed by AMTs is assimilated into amino acids via the glutamine synthetase/glutamine-2-oxoglutarate aminotransferase (GS/GOGAT) cycle and translocated to developing organs (Tabuchi et al., 2007). GS is a key enzyme in N assimilation and remobilization and forms the GS-GOGAT cycle to convert inorganic NH_4^+ into glutamine. Rice roots generally release oxygen from their aerenchyma, induce rapid nitrification in the rhizosphere, and take up NO_3^- at rates equivalent to those for NH_4^+ (Meng et al., 2019; Liu et al., 2022). A major bottleneck of NUpE improvement is the plastic nature of the root system. The N-induced mechanisms controlling root system architecture are more complex and diverse, with multiple regulation pathways operating depending upon both N forms and levels of N supply (Liu X et al., 2022; Liu Y et al., 2023). Increased rooting depth can be useful to uptake water and N from deeper layers of soil under stress conditions (Jia and von Wiren, 2020) and contribute to deep carbon sequestration to mitigate global warming and climate change. To date, there are more than 80 NRT1/PTR, four NRT2, and two NAR2 members in rice were discovered, but only a few of them have been clearly characterized (Li et al., 2018).

2.2. Nitrogen utilization

Nitrogen is utilized in many fundamental processes and is dependent on several climatic factors that otherwise have detrimental effects on crops (Xiong et al., 2018; Liu et al., 2023). Approximately 45–95 % of grain N in cereals comes from remobilized N stored in roots and shoots before anthesis (Hirel et al., 2007). Earlier, NUE has been enhanced by intensive selection of wheat genotypes for HI (Foulkes et al., 1998). However, despite N availability, HI is consistent in modern varieties (Barracough et al., 2010). Therefore, remobilization of N can be quite efficient in wheat, with little residual N remaining in the straw (Hawkesford, 2017). The improvement of N remobilization can be successful via changes in the stay-green traits that cause delayed senescence. The *ALKANE-RESPONSIVE ELEMENT ONE (ARE1)* gene encodes a chloroplast-localized protein, and its loss-of-function mutations conferred a 10–20 % increase in grain yield by modulating N utilization in rice (Wang et al., 2018a). Chen et al. (2020) applied a novel strategy for improving N remobilization to enhance the plant's NUE. The hypothesis behind this was based on the optimization of NO_3^- utilization and maintaining robust growth during N starvation of plants. Chen and colleagues (2020) introduced *AtNRT1.7* promoter-driven chimeric

hyperactive low-affinity NRT NC4N in *Arabidopsis*, tobacco and rice for improved NO_3^- remobilization from old to young leaves. The results indicated that increased source-to-sink nitrate remobilization could enhance NUE, biomass and grain yield. The plasticity of shoot branching and flowering in *Arabidopsis* may be accompanied by escape versus mitigate strategies for N utilization, which may be applied to improve the NUE of rice (de Jong et al., 2019). NUE can be derived from the ratio of grain yield by aboveground biomass N content (Good et al., 2004). Understanding factors behind the variability in N utilization efficiency is critical to improving NUE through breeding programs and agronomic management. The nonparallel increases in grain weight explain the reduction in NUE. Improving NUE can be significantly achieved by exploiting genotypic differences in diverse panels of rice populations. Rice breeders often do not include NUE as an ideotype factor to select and develop new varieties as NUE is a complex trait and highly regulated by multiple quantitative traits. Recently, many efforts have been made to study NUE. However, the genetic mechanism of NUE remains to be investigated further through utilization of non-invasive sensors technologies.

2.3. Physiological and phytohormone-mediated responses to low nitrogen stress

The knowledge of N-induced stress responses and phenotypic plasticity of canopy traits under the regulation of phytohormones is very crucial to developing N-efficient rice cultivars. Low N stress strongly affects the plant architectural traits (plant height, tiller number), including biomass and grain yield. N is a major growth-limiting factor as it constitutes around 1.5 to 2 % of the dry matter and 16 % of the total plant protein content (Frink et al., 1999). At the cellular level, N regulates the multiplication and expansion of the cell by negatively affecting DNA synthesis, cell division, cell growth, etc. Height and tiller number are two vital factors that shape the architecture of rice. The total N accumulation quickly increases, then achieves a plateau, and then slightly decreases during grain filling (Hashim et al., 2015). The number of effective tillers is strongly influenced by the rate of N uptake during the vegetative growth stages. After anthesis, the N used for seed setting is mainly remobilized from the culm and leaves (Hashim et al., 2015). This showed efficient N uptake and early canopy cover traits with optimal tiller number are very critical under N-starved condition. However, N sensing, uptake and shoot & root architecture development are highly modulated under the control of phytohormones.

In rice, the impact of N in architecture and its association with phytohormone-mediated signaling pathways have been recently reviewed (Luo et al., 2020). The impact of N limitation on growth and development is strongly regulated by multiple phytohormones such as auxin, cytokinins (CKs), strigolactones (SLs) and gibberellins (GAs) and their crosstalk. In rice, decreased NO_3^- can down-regulate the auxin efflux carriers *PIN-FORMED (OsPIN)* gene expression and reduce auxin transport from shoot to root. This caused the enhanced accumulation of radioactively labelled indole-3-acetic acid (IAA) in leaves than in the shoot and roots (Sun et al., 2014). On the other hand, overexpressing *OsPIN1* and *OsPIN5b* showed the opposite in transgenic plants (Xu et al., 2005; Lu et al., 2015). This result may be due to alterations in auxin or CK-induced lateral root inhibition (Barbier et al., 2019). Biosynthesis and CK translocation are strongly linked to N availability during root and shoot development. Elevated CK concentration promotes shoot growth, limits root growth and influences plant height, shoot branching, flowering and grain production. Maeda et al. (2018) indicated that after nitrate treatment, a transcriptional cascade-mediated repression *NLP/NIGT1* controls hormone metabolism. *IPT3* and *CYP735A2* genes encode cytokinin hydroxylases and were expressed in *Arabidopsis*. Kamada-Nobusada et al. (2013) found that repression of *OsiPT4* in rice roots significantly reduces the N-dependent increase of CKs in the xylem sap and retards shoot growth despite a sufficient N supply.

SLs are a new class of phytohormones with major functions in shoot

branching (Gomez-Roldan et al., 2008; Ramlal et al., 2024). Sun et al. (2016) provided evidence that SLs are involved in the downstream of N-dependent rice growth and development. N stress consequently enhanced the endogenous SL accumulation along with the degradation of the D53 protein (repressor), Yoneyama et al. (2012) reported the lesser significant influence of supplied N on SL contents in the roots of red clover and alfalfa. A NIN-like protein *OsNLP4* binds to NRE cis-element in the promoter of nitrite reductase (*OsNiR*) and induces the expression of *OsNiR*, thereby enhancing N assimilation, maximum tiller & grain yield in rice (Yu et al., 2020; Yu et al., 2021). Interestingly, Yu et al., (2020) also found that overexpression of *OsNiR* significantly downregulated the expression of genes involved in SL synthesis, particularly *D10*, *D17*, *D27* and *MAX1*, along with *D3*, *D14* and *D53* genes involved in the signaling pathway. However, the regulation of tiller number by the interaction between NH_4^+ and D53 involved in SL signaling and other genes involved in SL biosynthesis and signaling still remains unknown.

GA is well utilized in hybrid rice seed production systems due to its major function in cell elongation. Exogenous application of GA enhances plant height but reduces the number of tillers in rice (Zhuang et al., 2019). Liao et al. (2019) found in rice that GAs trigger both the degradation of DELLA protein (*SLENDER RICE 1: SLR1*) that regulates stem elongation along with the degradation of tiller number regulator (*MONOCULM 1: MOC1*); which is required for the formation of axillary meristems (AM), leading to reduction of tiller number in rice. Mutation in rice *NGR5* (*NITROGEN-MEDIATED TILLER GROWTH RESPONSE 5*), a transcription factor that negatively regulates GA levels, showed the insensitivity of tillering habits to varied N supply. Thus, *NGR5* negatively affects the expression of SLs signaling the *D14* gene and *OsSPL14* and hence promoting enhanced tillering (Wu et al., 2020). Taken together, these studies supported the notion that NUE is positively correlated with tiller development, flowering stages and planting methods. Therefore, optimal tiller production under N deficit conditions is regarded as a key goal for NUE in rice. However, due to the complexity of N regulation pathways, it is difficult to predict the effect of N on architecture, grain yield and its components as selection criteria for crop improvement (Luo et al., 2020). Thus, more inclusive investigation strategies are required to understand the complex regulatory pathways of phytohormones for N regulation that influence rice plant architecture.

2.4. Strategies for improving nitrogen use efficiency

The high rate of N fertilizer application significantly increased input costs and environmental pollution. Breeding for high NUE crops is imperative to achieve more gains per unit fertilizer input and thus reduce input cost and environmental pollution. Current toolboxes and future perspectives for improving NUE in rice have been proposed by Ali et al. (2018) and Zhang et al. (2020). Improvement of NUE required two-pronged strategies viz., 1) improving agronomic practices and 2) genetic improvement through conventional breeding, transgenic and genome editing.

Firstly, NUE can be improved through best agronomical management practices, including slow-release fertilizers, root-microbiota interaction, reducing soil pH, improving soil drainage, and proper canopy size management (Sharma and Bali, 2018; van Bueren and Struik, 2017). In slow-release fertilizer, the Government of India adopted neem-coated urea technology in 2015, which directly saved 10 % urea as neem-coated urea was prepared as a 45 kg bag while conventional urea was prepared as a 50 kg bag. Besides, it improved the NUE significantly. Matching the N fertilizer application with the crop demand at different physiological stages can significantly improve NUE. Reduction of N application rate without incurring yield penalty is one of the major agronomical approaches. In Australia and China, the N application rate has been reduced by 20 % with no yield penalty (Dong et al., 2010; Rochester et al., 2009; Anas et al., 2020). NUE in crops can be improved based on canopy management through a hand-held normalized

difference vegetation index (NDVI) meter (Reynolds et al., 2020). The principle behind this is the greenness index and chlorophyll content can indirectly estimate plant N through the ratio of fluorescence signals at 690 and 730 nm (Amaral et al., 2015). Rubisco acts as the major sink of N and strongly correlates with chlorophyll content. Therefore, the ratio between F690 and F730 acts as a surrogate for the N content. Interestingly, simple Leaf Color charts have also been shown to be effective in improving NUE; for example, IRRI leaf color charts and Punjab Agricultural University, India leaf color charts assist in decision-making on the timing and quantity of N fertilizer application (Swarbreck et al., 2019). The result has shown a significant reduction in N application, approximately 75 kg N ha⁻¹ in rice (2017) and 50 kg N ha⁻¹ in wheat (2017–2018).

Secondly, NUE can be improved through genetic approaches. Ali et al. (2018) and Hawkesford and Griffiths, (2019) have reviewed recent advances in genetic approaches for NUE breeding in cereals. Identifying the QTL/genes associated with NUE by genetic mapping is a vital first step in its improvement. Several candidate QTLs/genes have been mapped in rice (Wei et al., 2012; Vishnukiran et al., 2020), wheat (Xu et al., 2014; Brasier et al., 2020), maize (Li et al., 2015) and barley (Mickelson et al., 2003; Han et al., 2016). High genetic variability of NUE has been observed in cereals, especially under N limitation (Hirel et al., 2007; Hawkesford & Griffiths, 2019). A QTL named *qNGR9* synonymous with *DEP1* responsible for plant height response to N has been identified, and the plants carrying the dominant *dep1-1* allele displayed increased N uptake and assimilation, resulting in improved HI and grain yield (Huang et al., 2009; Sun et al., 2014). A major QTL Tolerance Of Nitrogen Deficiency 1 (TOND1) located in chromosome 12 was mapped for nitrogen deficiency tolerance in the *indica* cultivar Teqing. Further transgenic overexpression of TOND1 enhanced the N-deficiency tolerance of the susceptible rice genotype (Zhang et al., 2015). Similarly, by using a chromosomal segment substitution line (CSSL) population, a major QTL *qRDWN6^{XB}* for N-deficiency tolerance was mapped in the *indica* variety XieqingzaoB, and *LOC.Os06g15910*, a potassium transporter, was identified as putative candidate gene (Anis et al., 2019). Similarly, Hu et al. (2023) have reviewed QTLs involved in NUE in rice.

Genetic manipulation of genes associated with N-regulation can enhance the NUE and grain yield. These genes mainly encode ammonium and NRTs, and enzymes of N metabolism (Good et al., 2004). Many studies have been focused on the investigation of the natural variations of N transporters. The variation in the *NRT1.1B* (*OsNPF6.5*) gene contributes to nitrate-use divergence between *indica* and japonica rice varieties (Hu et al., 2015). *Indica* varieties usually have higher NUE than japonica. *NRT1.1B* was again confirmed as a major factor explaining the difference in NUE between *indica* and japonica by being associated with root microbiota (Zhang et al., 2019). A recent study has discovered that expression of a NRT gene encoding tonoplast localized *NRT1.1A/NPF6.3* in japonica confers high yield and early maturation due to the upregulation of the expression of N utilization-related genes (Wang et al., 2018b; Wang et al., 2018d). The *ARE1* gene encodes a chloroplast-localized protein, and its loss-of-function mutations exhibited delayed senescence and enhanced grain yield by 10–20 % through modulation of NuTE in rice (Wang et al., 2018a). Tang et al. (2019) identified *OsNPF6.1* and *OsNAC42* associated with effective panicle number ratio (EPNR) and plant height ratio (PHR), respectively based on GWAS of 117 rice accessions. The elite haplotype (*OsNPF6.1^{HapB}*) was shown to enhance the transport activity. However, it was found that *OsNPF6.1^{HapB}* is not present in 90.3 % of rice varieties, suggesting its potential for introgression in elite varieties for enhanced NUE. *OsNR2* encoding an NADH/NADPH-dependent NO₃⁻ reductase (NR) was found to confer an improved number of tillers, NUE and grain yield per plant (Gao et al., 2019). In addition, the concentration of cytosolic nicotinamide adenine dinucleotide glutamate synthase (NADH-GOGAT) and GLS1 enzymes involved in N metabolism was enhanced to improve the NUE. Earlier, a similar response of a QTL region was identified from offsprings of backcross between Nipponbare (japonica) and Kasalath

(aus) (Obara et al., 2001; Yamaya et al., 2002).

Thirdly, NUE can be improved by transgenic approaches. The attempts to improve NUE through transgenic approaches have targeted many N sensing and signaling, transporters, remobilization amino acid biosynthesis, and homeostasis-related genes (McAllister et al., 2012; Garnett et al., 2015). James et al. (2018) demonstrated an increase in the grain yield under N stress conditions through concurrent overexpression of *OsGS1* and *OsGS2* genes in rice. Yamaya et al. (2002) attempted to increase the grain weight by 80 % by overexpressing NADH-GOGAT. The most promising improvement of NUE through transgenic approaches has been reported by overexpressing the alanine aminotransferase (AlaAT) gene (Good et al., 2007; Shrawat et al., 2008), glutamate dehydrogenase (GDH) (Abiko et al., 2010), GLS (Brauer et al., 2011), the rice nitrate transporter NRT2.3/2.5 (Fan et al., 2016). Regardless of the tireless effort to improve the NUE through both conventional breeding and transgenic approaches, only very limited success has been achieved in the varietal development program. In recent years, several QTL/genes have been identified that can be used to enhance plant NUE. Equipped with low-affinity and high-affinity nitrate transporters, plants can optimize nitrate utilization and maintain energetic growth during N starvation. Chen et al. (2020) employed a genetic engineering strategy in *Arabidopsis*, tobacco and rice by introducing a hyperactive chimeric nitrate transporter driven by the promoter of *AtNRT1.7* that encodes a low-affinity nitrate transporter responsible for nitrate remobilization. The transgenic plants increased source-to-sink nitrate remobilization and enhanced NUE and yield. Their result established a novel approach for improving N remobilization to improve the plant's NUE, growth and yield.

Together, these studies indicate that NUE is a complex trait governed by multiple interactive cellular processes. Available N from the soil is taken up and translocated into the root, assimilated and utilized to produce biomass and remobilize N to the grain. Unfortunately, traditional breeding based on promising NUE lines has not been successful. Hawkesford and Riche (2020) suggested having multi-location and/or years of field trials to reduce the influence of environmental components between genotype and phenotype. Approaches to improve rice NUE have focused diversely on the genetic manipulation of N uptake, metabolism, partition, assimilation and remobilization. All of these studies are normally successful under specific N conditions. However, most studies did not account for the different NUE components and/or the effects of different N environments on NUE. Most studies only showed that NUE, growth and grain yield were improved when transgenic plants were grown under a specific N condition. Hence, NUE is a quantitative complex trait, and these regulation and inheritance mechanisms are not fully understood.

3. Phenomics: Next-generation plant phenotyping and its applications for improving nitrogen use efficiency

3.1. Crop phenomics, genomic selection and high-throughput phenotyping

HTP platforms used for non-destructive phenotypic measurements combine various types of sensors and computer science applications in both field and control environments (Furbank and Tester, 2011; Kumar et al., 2016; Furbank et al., 2019; Yang et al., 2020). HTP methodology offers breeders a tool for precision phenotyping of resource use efficiency and deciphering genotype-by-environment interactions (Watt et al., 2020). Modern phenomics with precise HTP is a promising tool to elucidate genotype-by-environment interactions and speed up plant genetic improvements (Hansen et al., 2018; Yang et al., 2020). Manual phenotyping data collection is time-consuming, with low accuracy at individual time points. In the last decade, recent advances in sensors, machine vision, automation technology and mathematical algorithms revolutionized plant phenotyping. The rapid advancements in remote sensing and statistical methods have made significant contributions to the precision phenotyping of plants for N-related studies (Fahlgren et al.,

2015a,b; Nguyen and Kant, 2018; Banerjee et al., 2020; Moreira et al., 2020). Popular controlled-environment phenotyping platforms have been reported, such as the Scanalyzer 3D (Lemna Tec) imaging system, in which plants are located on a conveyor system with automated scheduling of watering and transferred to sensors for automated image acquisition (Neilson et al., 2015; Elangovan et al., 2023). Banerjee et al. (2020) reported the application of digital multi and hyperspectral imaging sensors to develop a novel vegetation index for estimating chlorophyll content and monitoring the N responses.

The new era of HTP platforms provided strong evidence for improving genomic selection (GeS) in cereals. Several HTP studies have shown that selection accuracy can be increased by using a multi-trait strategy, including primary and secondary phenotypic traits measured during the early stage, such as canopy temperature (CT) and NDVIs with genomic breeding values for yield. Rutkoski et al. (2016) used aerial HTP platforms to measure CT and green and red NDVI (G- & R- NDVI) from 557 diverse wheat lines to predict grain yield. These secondary traits could significantly increase prediction accuracies for grain yield in the genomic prediction models. Watanabe et al. (2017) employed a UAV carrying a remote-sensing unit with both RGB and NIR green and blue (NIR-GB) cameras for the HTP measured the height of 115 sorghum germplasm accessions and evaluated phenotypic traits in a GeS model. Even though the phenotypic correlation was not high, these results provided a potential with low cost and easy operation of HTP tool for genomics-assisted crop improvement. Breeders always wish to select the inbred lines or genetic materials with high genomic breeding values for the next selection season. Combining HTP data with genotypic information is useful to improve GeS for grain yield in wheat. Crain and colleagues, (2018) at the International Maize and Wheat Improvement Center (CIMMYT) combined dynamic HTP data extracted from PhenoCart (CT, NDVIs) with 2254 GBS markers derived from 1170 wheat lines under drought and heat stress for GeS and GP. Based on the heritability of traits and correlation to grain yield, these traits showed great potential for increasing the prediction accuracy and later could be used for the selection of grain yield under abiotic stresses.

3.2. HTP phenotyping facilities and longitudinal phenotypic traits

In phenomics, longitudinal traits refer to the phenotypic characteristics of plants that are measured repeatedly over time, capturing their development and responses to environmental conditions throughout their lifecycle. The beauty of HTP is that it can quantify the longitudinal traits or infinite dimensional traits (Kirkpatrick and Heckman, 1989) or function-valued traits (Promislow et al., 1996) throughout the life cycle to monitor its response to the environment (Yang et al., 2006). In plants, it is most important to dissect the complex NUE traits, which are polygenic traits for understanding the genetic mechanisms of time-specific QTLs/genes. Campbell et al. (2018) implemented a random regression approach for genomic prediction of longitudinal shoot growth trajectories in rice. Momen et al. (2019) applied random regression models to forecast shoot growth trajectories using B-splines and Legendre polynomials in well-watered and water-limited conditions under various longitudinal cross-validation scenarios. Moreira et al. (2020) highlighted current statistical genetics and modeling to improve longitudinal traits in crops. From information on wheat field-based HTP systems, Lyra et al. (2020) identified several temporal QTLs associated with plant height. However, the phenotypic plasticity of NUE in rice is very scarce. Therefore, underpinning the relationships between genetic makeup and the dynamics of phenotypic expression requires greater integration of high throughput precision phenotyping, molecular technologies and statistical methodology.

HTP facilities around the world have been well-reviewed by Zhao et al. (2019), An et al. (2020) and Watt et al. (2020). Briefly, the phenotyping system use the Scanalyzer3D platform and related image processing software developed by LemnaTec GmbH (Germany). Commercial LemnaGrid software for image processing and analysis has

built-in algorithms and cannot be easily modified to extract more traits. There is a bottleneck in phenotyping method quantification for various crop responses to different treatments and stress levels. Some years ago, a group of Chinese scientists developed an HTP system amalgamating red–green–blue (RGB), X-ray CT sensors and an automatic image processing pipeline to capture multiple phenotypic variations of complex physiological traits in rice, sorghum and maize (Yang et al., 2014; Zhang et al., 2017; Duan et al., 2018; Guo et al., 2018). Integrated Analysis Platform (IAP) was developed by a group of German scientists at the Leibniz Institute of Plant Genetics and Crop Plant Research, Gatersleben (Klukas et al., 2014). It is a Java-based open-source, supports multiple sensors (RGB, fluorescence, near-infrared, infrared) with a broader range of species (maize, wheat, barley and *Arabidopsis*), and expedites data management. IAP extracted features from images and model-derived parameters to determine drought response and predict barley biomass (Chen et al., 2014; Chen et al., 2018). Muraya et al. (2017) combined the power of HTP and GWAS to investigate the genetic variation and growth dynamics in maize. Donald Danforth Plant Science Center (USA) has developed a low-cost HTP platform, PlantCV. Open-source libraries like OpenCV, NumPy, and Matplotlib are used to process the RGB, NIR and Fluorescence images (Fahlgren et al., 2015a,b; Gehan et al., 2017). PlantCV has been used to capture multiple phenotypic variations in foxtail millet (Fahlgren et al., 2015a,b) and maize (Enders et al., 2019). Nowadays, there are many options for plant breeders to perform HTP from lab to field. Reynolds et al. (2020) presented a “breeder-friendly” phenotyping. The researchers can select the suitable phenotyping method to collect data easily and cost-effectively. The HTP facilities and current application status in India are shown in

Table 1
High-throughput phenomics facilities in India.

Name	Establishment year	Capacity
Phenomics facility, ICAR - Central Research Institute for Dryland Agriculture (ICAR – CRIDA), Hyderabad	1 July, 2014	+ Scanalyzer system (LemnaTec) + 240 carts + 3 sensors: RGB, NIR, IR + Automate weighing & watering
Phenomics facility, ICAR - Indian Institute of Horticultural Research (ICAR-IHR), Bengaluru	1 November, 2015	+ Scanalyzer system (LemnaTec) + 240 carts + 3 sensors: RGB, NIR, IR + Automate weighing & watering
Phenomics facility, ICAR - National Institute of Abiotic Stress Management (ICAR-NIASM), Baramati	23 October, 2016	+ Scanalyzer system (LemnaTec) + 240 cars + 3 sensors: RGB, NIR, IR + Automate weighing & watering
Nanaji Deshmukh Plant Phenomics Centre, ICAR – IARI, New Delhi	11 October, 2017	+ Scanalyzer system (LemnaTec) + 1200 cars + 8 sensors: 1. Thermal IR imaging (8000–14000 nm) 2. Chlorophyll fluorescence PSII imaging 3. Imaging unit for additional sensors 4. NIR (900–1700 nm) & RGB root imaging 5. Visual RGB color imaging (400–700 nm) 6. NIR shoot imaging (900–1700 nm) 7. Hyperspectral imaging (VNIR 400–1000 nm and SWIR 1000–2500 nm) + Automate weighing & watering

Table 1. The journey of HTP in India has started in 2014. To date, very few papers highlighting the function of HTP facilities have been released. It showed a gap in utilizing HTP facilities and mainly linked to image processing, image analysis, data processing and data management. Therefore, it is urgently required to integrate multidisciplinary teams working together.

Nanaji Deshmukh Plant Phenomics Centre (NDPPC), ICAR – IARI, New Delhi, India, (Indian Council of Agricultural Research, India (ICAR) through the National Agricultural Science Fund, India (NASF)) contains four climate-controlled greenhouses equipped with 1200 plant carriers with RFID chip tags (Table 1; Fig. 1). The plant carrier on the moving field conveyor system randomizes plants within the greenhouse and carries them for automated weighing, watering, and imaging at various imaging platforms. Eight different imaging platforms were established such as visual RGB color imaging; thermal infra-red (8000–14000 nm) imaging; near infra-red shoot imaging (900 to 1700 nm); chlorophyll fluorescence imaging; unit with a calibrated light source (for optional sensors – spectroradiometer/FTIR); root imaging - near infra-red (900 to 1700 nm) & visible color imaging; visible-near infra-red hyperspectral imaging (400–1000 nm); and shortwave infra-red hyperspectral imaging (1000–2500 nm). NDPPC is the largest in India and one of the best facilities in terms of analytical capabilities among publicly funded institutions throughout the world. The major function of HTP is to capture the maximum phenotypic variation in crops and provide valuable information for the breeding programs (Fig. 2). Therefore, multidisciplinary team collaboration from all fields such as genetics, plant breeding, physiology, and biochemistry to bioinformatics and computer science is required to enhance productivity and efficiency cost-effectively. Most of the HTP platforms worldwide are of two types viz., controlled environmental and field phenotyping facilities.

The merits of the HTP systems are mainly attributed to increased throughput, the precision of trait collection, spatio-temporal-trait resolution, non-destructive/ non-invasive trait measurement and convenience of automatic collection of phenome-wide trait collection. Through a collection of multi-dimensional images, the major advantage of HTP platforms is related to archiving phenome image databases for the development of immortal data and in-silico phenotyping in the future. The major demerits of HTP platforms include 1) the complexity of technology (big data handling, image data processing, multi-traits analytics, interpreting biological significance etc.), 2) the high initial investment and 3) maintenance costs.

3.3. Image processing and analysis

An image-processing pipeline consists of four key steps: pre-processing, segmentation, feature extraction and post-processing (Perez-Sanz et al., 2017; Duc et al., 2023). Marko et al. (2018) provided an overview of a huge collection of software tools, plant, crop models and datasets for plant image analysis to extract a wide range of measurements, such as root system architectures (RSA), canopy architecture, crop growth rate and leaf and seed traits (<https://www.quantitative-plant.org/>). However, the capacity to capture the phenotypic variation from these tools is still very limited. Many phenomics facilities worldwide were established by LemnaTec Scanalyzer 3D platform (LemnaTec GmbH, Aachen, Germany) with LemnaGrid software for image processing. It is quite efficient for systematically capturing the whole phenotypic traits and was successfully used in the prediction of biomass accumulation for *Arabidopsis* (Arvidsson et al., 2011; Camargo et al., 2014) and cereals (Campbell et al., 2015; Al-Tamimi et al., 2016; Chen et al., 2018; Nguyen et al., 2019a,b). However, LemnaGrid is commercial software, highly expensive, and has the limitations of extensibility and customization by the user (Berger et al., 2012; Knecht et al., 2016). To tackle this bottleneck, several open-source tools have been developed, such as HTPheno – an ImageJ plugin (Hartmann et al., 2011), Plant CV - a Python platform (Fahlgren et al., 2015a,b; Gehan et al., 2017), IAP – a GUI-based platform (Klukas et al., 2014), Image

Table 2
Formulas and definitions used to demonstrate nitrogen use efficiency in plants.

Name	Abbreviation	Definition	Calculation	Unit	References
Nitrogen uptake efficiency	NUpE	The efficiency of absorption/uptake of N supply	Total N in plant / (N supply + N available in soil)	g/g	Moll et al. (1982)
Nitrogen utilization efficiency (Internal efficiency)	NUtE	The efficiency of assimilation and remobilization of plant N to produce grain.	Grain weight / Total N in plant	g/g	Moll et al. (1982)
Nitrogen harvest index	NHI	The ratio of grain N to the total N uptake at harvest	Grain N / Total N in plant	g/g	Good et al. (2004); Rao et al. (2018)
Physiological nitrogen use efficiency	PNUE	Measures the efficiency of capture of plant nitrogen in grain yield	(Grain weight of plant in Control pot - Grain weight of plant under N-deficit stress) / (Total N in plant in Control - N in plant in N stress)	g/g	Rao et al. (2018)
Nitrogen use efficiency	NUE	Measures the Carbon: Nitrogen ratio of the plant	Shoot weight in dry weight / N content of dry shoots	g/g	Steenbjerg and Jakobsen, (1963)
Nitrogen Use efficiency	NUE Wei2012	Measure the relative change in N content in plant stress by control	(Total N contents of plants in the Control - Total N contents of plants in the N stress) / (Total amount of N applied + N available in initial soil)	%	Wei et al. (2012), Yang et al. (2017)
Nitrogen Use Efficiency per pot in term of biomass	NUEb	Biomass per (nitrogen supply and available N in initial soil)	Biomass / N (supply + available N in initial soil)	g/g	Nguyen et al. (2019a,b)
Nitrogen Use Efficiency per pot in term of grain yield	NUEg	Grain weight per (N supply and available N in initial soil)	Grain weight / N (supply + N available in initial soil)	g/g	Nguyen et al. (2019a,b)
Nitrogen uptake efficiency susceptibility index	NUESI	(N percentage in plant under Control - Nitrogen percentage in plant under N-deficit stress) / N % in plant under Control	[(N% in plant in Control) - (N% in plant under N stress)] / (N% in plant under Control)	%/%	new proposed



Fig. 1. Nanaji Deshmukh Plant Phenomics Centre (NDPPC), ICAR – IARI, New Delhi, India. (A) Panorama view of the Centre; (B) Plants are grown in a greenhouse with conveyor belts; (C) Movement of plants in an automated system and weighing and watering. (D) Imaging chambers to capture different types of data, (E) An example of the side view RGB image of a rice.

Harvest - a python platform (Knecht et al., 2016). Despite the power and flexibility of these tools, it is still challenging for many plant physiologists and plant breeders with limited or no computer programming skills. Therefore, LemnaGrid software is still an optimum choice for processing and extracting multi-dimensional HTP image traits.

3.4. HTP data collection, processing and analysis

Analysis of big data generated by HTP platforms is a major challenge for plant science scientists. It is quite complex to handle, process, and analysis. A data analysis pipeline is required to dissect phenotypic variation. Although there is an explosion of HTP systems developed for

plant phenomics, the phenotypic variances underlying dynamic processes such as growth, development, or responses to environmental challenges and their properties, are rarely found in the literature. The data processing and analysis pipeline consists of five major components: experiment design, image acquisition, data management, image processing and data analysis (Chen et al., 2014; Perez-Sanz et al., 2017). Briefly, the experimental setup can be controlled to minimize the effect of the environment on the greenhouse system. The intensity of stress, the level of irrigation and the content of nutrients can be defined and controlled during a phenotyping experiment. Various types of image data, such as visible (VIS), near-infrared (NIR), infrared (IR), visible and near-infrared (VNIR) hyperspectral, short-wave infrared (SWIR)

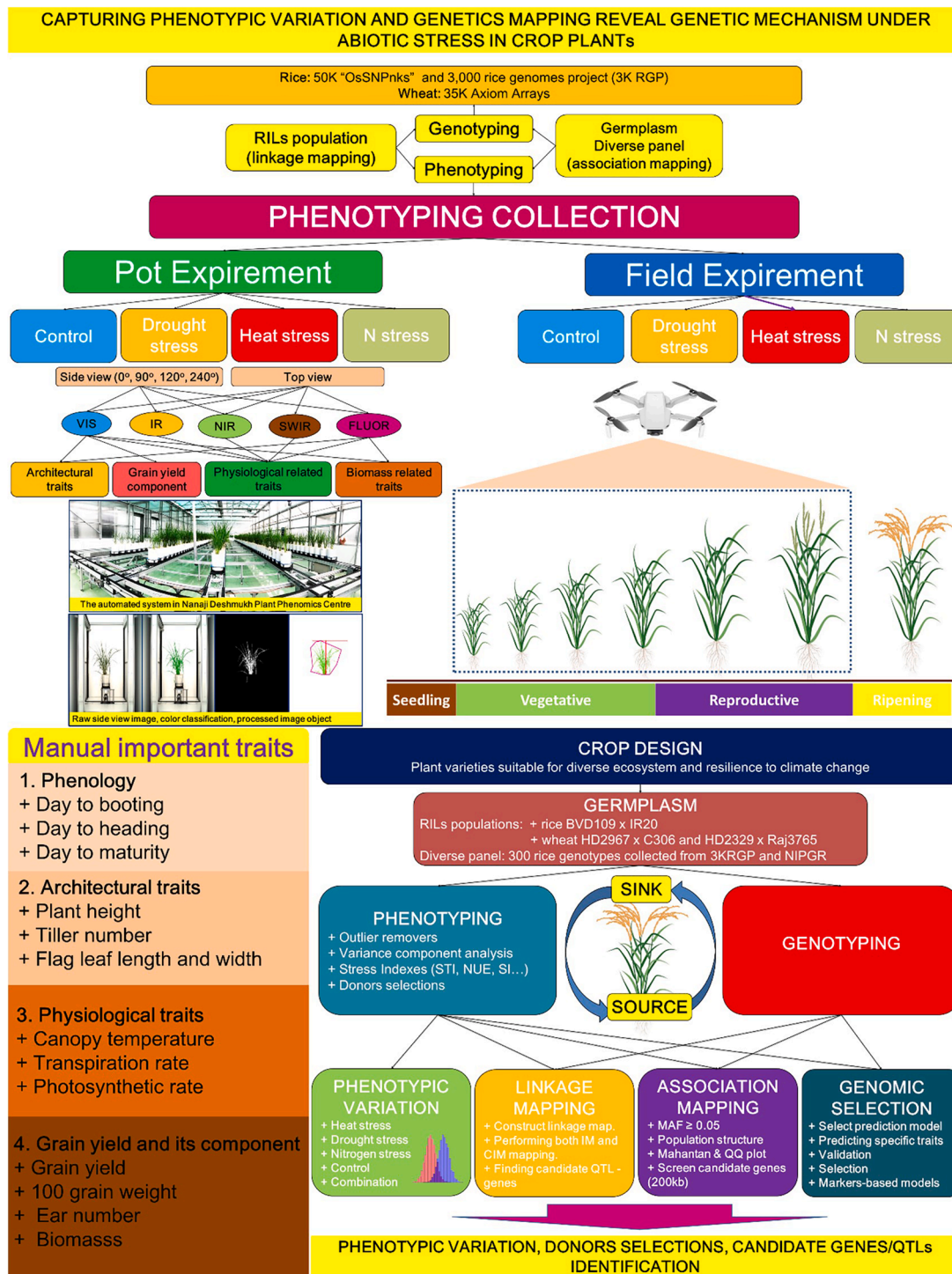


Fig. 2. Workflow to capture phenotypic variation and genetic mapping to dissect genetic mechanisms under abiotic stress in crops.

hyperspectral, and fluorescence (FLUOR) images, can be acquired daily/hourly from different views (top view and side views from different angles) in the phenotyping platform. Consequently, timely retrieved data from the imaging system are organized into a data management system and subjected to the automated image processing pipeline that extracts many phenotypic trait values. Finally, by applying well-established statistical models, the extracted phenotypic traits can be used to assess plant growth and performance features. [Chen et al. \(2014, 2018\)](#) provided a role of statistical model for investigating the HTP data effectively. [Van Eeuwijk et al. \(2019\)](#) suggested a pipeline for HTP analysis to obtain significant information to understand natural

biological processes. Many efforts have been made in HTP development, reducing phenotyping costs and laboriousness. Automated HTP platforms designed for control environment and field conditions support breeders to capture the dynamic longitudinal traits. The novelty of HTP is non-destructive and continual measurements over time. Recently, machine learning (ML) and deep learning are emerging as promising tools to connect phenotypic and genotypic big data ([van Dijk et al., 2021](#); [Tong and Nikoloski, 2021](#)). Furthermore, by integrating data from other domains, these image-based traits and model-derived parameters are promising for genetic mapping, functional analysis and GS. Precision phenotyping is urgently required to improve the detection of complex

traits with small effects QTLs. Modern crop improvement and plant breeding in the 21st century will require multidisciplinary teams consisting of researchers from biology, bioinformatics, genetics, computer science, sequencing, data analysis, mathematic models and statistics in order to solve the world food security challenges for sustainable development.

4. Genome-wide association study in rice

4.1. GWAS in rice

GWAS in rice has been used to identify many candidate genes associated with not only agronomical important traits, both manual and HTP collection, but also biochemical and molecular phenotypes. GWAS models are summarized in Table 3 and statistical model comparison is provided in Table 4. The first application of GWAS in rice as a model crop was made in 2010, and the first use of WGRS for constructing a haplotype map was also in rice (Huang et al., 2010). The authors used approximately 3.6 million SNPs of 517 rice indica landraces to study 14 agronomic traits using compressed mixed-linear models (CMLM). Significant QTLs explained about 36 % of the phenotypic variation, and QTLs for six traits were located close to previously identified genes (*OsC1*, *Rc*, *ALK*, *Waxy*, *qSW5*, *GS3*). The diversity of *O. sativa* accessions throughout the world can be classified into 7 sub-groups indica, aus, tropical japonica, temperate japonica, aromatic and admixed sub-populations (Zhao et al., 2011). Huang et al. (2012) conducted sequence-based GWAS in rice using 950 varieties and identified 32 new loci related to flowering time and grain traits. Zhao et al. (2011) generated 36,901 SNPs (44 K) across 413 accessions in all rice sub-populations from 82 countries and used it to study association with 34 traits containing physiological, developmental and morphological traits using the EMMA eXpedited (EMMAX) model.

McCouch and colleagues (2016) provided a valuable resource for GWAS studies in rice. They used a high-density rice array across 1,568 rice accessions throughout the world to generate 700,000 SNPs (approximately 1 SNP in 0.54 kb) (700 K). The 700 K SNP dataset in the panel was used to demonstrate the improving power of GWAS to detect major and minor effect QTLs for grain length. GWAS based on whole genome resequencing uncovered four major genes viz., *LOC_Os01g62780*, *LOC_Os11g08410*, *LOC_Os04g52479* and *LOC_Os08g37890* associated with days to heading, plant height & panicle length, panicle number & spikelet number per panicle, and awn length, respectively (Yano et al., 2016).

In recent years, cost-effective genotypic platform C7AIR (an improved 7K SNP array) containing 7,098 single nucleotide polymorphic (SNP) markers and 1k-RiCA (1K-Rice Custom Amplicon) containing ~1,000 SNP markers with an average distance of 1.5 cm between SNPs were developed for marker-assisted selection (MAS) and GeS in rice breeding programs (Morales et al., 2020). These SNP data are available as valuable resources for diverse genetic analysis, including genome-wide association study (GWAS) studies in rice. In 2015, a low-cost 50K SNP chip, "OsSNPnks," was designed by Affymetrix Axiom Technology, suitable for genetics and breeding applications in rice (Singh et al., 2015; Bollinedi et al., 2020; Krishnamurthy et al., 2020; Rohilla et al., 2020). "OsSNPnks" has been utilized by many researchers for mapping resilience to abiotic and biotic stress and improve resource use efficiency in rice (Bollinedi et al., 2020; Hada et al., 2020; Rohilla et al. 2020). Bollinedi et al. (2020) applied GWAS with the FarmCPU model and identified 29 significant MTAs associated with Zn and Fe across 192 Indian rice accessions. GWAS with MLM model was performed with 272 rice accessions to discover the unique sources and genetic basis of rice root-knot nematode resistance. Later, a total of 17 novel SNPs significantly associated these phenotypic traits harboring the candidate genes, including NBS-LRR, Cf2/Cf5 resistance protein, MYB, bZIP, ARF, SCARECROW and WRKY transcription factors were discovered (Hada et al., 2020). GWAS with MLM model was applied across 94

Table 3
GWAS models.

Model	Description	Features	Software	References
MLM	Mixed Linear Model	Control both population structure (Q) and kinship (K). Add Q, K or Q+K matrix with traditional mixed model.	TASSEL, PLINK1.9, GEMMA, EMMAX, R package rMVP & package GAPIT3	Yu et al. (2006), Wang and Zhang (2021), Yin et al. (2021)
GLM	General Linear Model	Incorporate either the Q matrix or PCs as covariates. Use Q+K model.	TASSEL, R package "rMVP"	Yin et al. (2021)
EMMA	Efficient Mixed Model Association	Estimate variance components in each test.	R package "emma"	Kang et al. (2008)
EMMAX	EMMA eXpedited	Modified from EMMA to run tens of thousands of individuals at hundreds of thousands of SNPs. Time efficient.	EMMAX	Kang et al. (2010)
CMLM	Compression Mixed Linear Model	Increase statistical power by 5–10 % and reduce computing time from weeks to hours.	TASSEL, R package "GAPIT3"	Zhang et al. (2010)
FaST-LMM	FaST linear mixed models	Scales linearly with cohort size in both run time and memory use.	R package "GAPIT3"	Lippert et al. (2011)
MLMM	Multi-Locus Mixed Model	Based on EMMAX and multi-locus. Use multiple cofactors in the statistical models.	R package "GAPIT3"	Segura et al. (2012)
GEMMA	Genome-wide Efficient Mixed Model Analysis	Based on EMMA, but reduce times using the usual genome-wide relatedness matrix (with run time close to EMMAX)	GEMMA	Zhou and Stephens (2012)
ECMLM	Enriched CMLM	Improve statistical power by selection of the markers according traits of interest.	R package "GAPIT3"	Li et al. (2014)
SUPER	Settlement of MLM Under Progressively Exclusive Relationship	Derived kinship from associated markers (QTNs) instead of all markers and use QTNs that are complementary to testing markers.	R package "GAPIT3"	Wang et al. (2014)
FarmCPU	Fixed and random model Circulating Probability Unification	Select associated markers as cofactor to control false positives using likelihood in MLM to avoid overfitting.	R package "rMVP" & package "GAPIT3"	Liu et al. (2016), Kusmec and Schnable (2018)
BLINK	Bayesian-information and Linkage-disequilibrium Iteratively Nested Keyway	Multiple loci model, higher statistical power than MLM	R package GAPIT3	Huang et al. (2019)

Table 4
Comparison of statistical models used in GWAS.

Function	Items	PLINK 1.9	GEMMA	TASSEL	GAPIT 3	rMVP	
Model	MLM	yes	yes	yes	yes	Yes	
	GLM	no	yes	yes	yes	Yes	
	EMMA	no	no	yes	yes	No	
	EMMAx	no	no	no	yes	No	
	CMLM	no	no	no	yes	No	
	FaST-LMM	no	no	no	yes	No	
	MLMM	no	no	no	yes	No	
	ECMLM	no	no	no	yes	No	
	SUPER	no	no	no	yes	No	
	FarmCPU	no	no	no	yes	Yes	
	BLINK	no	no	no	yes	No	
	Input	Hapmap	no	no	yes	yes	Yes
		VCF	yes	no	yes	yes	Yes
		BIMBAM	no	yes	yes	no	No
Numeric		no	no	yes	yes	Yes	
Binary		yes	yes	yes	no	Yes	
Features	Quality control	yes	no	yes	yes	No	
	Multiple loci	no	no	yes	yes	Yes	
	Multiple traits	no	yes	yes	yes	Yes	
	Genomic selection	no	no	yes	yes	No	
	Computer program language	C++	C++	Java	R	R	
	User interface	Command line and GUI	Command line	Command line, GUI and R package	R package	R package	
	Data management	yes	yes	yes	no	No	
VCE	BRENT	no	no	no	no	Yes	
	EMMA	no	no	yes	yes	Yes	
	Fast-LMM	no	no	yes	yes	Yes	
	HE regression	no	yes	no	no	Yes	
PS	Principal components	yes	no	yes	yes	Yes	
	Genomic relationship matrix	no	yes	yes	yes	Yes	
Output	Manhattan plot	no	no	yes	yes	Yes	
	QQ-plot	no	no	yes	yes	Yes	
	Phenotype distribution	no	no	yes	yes	Yes	
	PCA plot	no	no	yes	yes	Yes	
	SNP density plot	no	no	no	no	Yes	
	Effect, SE, p-values	yes	yes	yes	yes	Yes	

Note: VCE: Variance components estimation; PS: population structure; GUI: graphical user interface

deep-water rice genotypes of Assam, and 20 significant SNPs with two major genes, *OsXDH* and *SSXT*, associated with anaerobic germination-related traits were identified (Rohilla et al., 2020).

In 2018, the 5,231,435 (5.2 M) SNPs with no missing SNPs have been imputed on the 3,010-rice panel by comparing the 700K SNPs with whole genome sequence data of 3,000 sequenced rice cultivars (Wang et al., 2018c). The 5.2M SNP data were used by those authors for GWAS of grain amylose content, revealing the major loci associated with the trait. Related to NUE research in recent years, Tang et al. (2019) applied the GWAS approach to identify seven NUE-associated genes and two novel genes, *OsNPF6.1* and *OsNAC42*, that confer NUE. Wang et al. (2020) have highlighted the major advantages of GWAS over linkage mapping in studying the complex traits in rice. For instance, GWAS exploits the high genetic diversity and mapping resolution present in the diverse population than in linkage mapping performed in the bi-parental mapping population. GWAS can be applied as a vital approach to identify multiple loci harboring causal variants contributing to agronomic traits. In the next decade, GWAS will accelerate marker-trait associations to discover key genes and alleles associated with NUE traits in major crops (Xu & Takahashi, 2020).

4.2. Candidate QTLs/genes associated with NUE in rice

NUE is a complex physiological trait and is quantitatively controlled by many major genes and minor effects QTLs. Whole genome scanning of genes/QTLs, cloning and functional analysis have provided important fundamental knowledge on the physiological and molecular mechanisms conferring rice NUE. Sun et al. (2014) mapped a major NUE QTL, *qNGR9* (LOC_Os09g26999), in rice, which regulates the activity of G protein in response to nitrogen. One another major NUE QTL mapped on

chromosome 12, *TONDI* (LOC_Os12g43440) and found that its over-expression enhanced the tolerance of rice to nitrogen deficiency (Zhang et al., 2015). Yang et al. (2017) used rice recombinant F2 population from a cross between NIL-13B4 and GH998 to study NUE. A major candidate QTL *qNUE6* with 266.5kb (LOC_Os06g15370 and LOC_Os06g15420) was mapped. Vishnukiran et al. (2020) used 291 RILs developed between BPT5204 (high quality) and PTB1 (high NUE) to study NUE in rice. A major stable pleiotropic QTL (RM13201-RM13209) with 825.4 kb located in chromosome 2 was found and considered promising for the MAS. GWAS analysis has successfully provided candidate genes related to NUE in rice. Tang et al. (2019) applied GWAS approach in a rice population in three years field experiment under high and low N level and identified seven NUE-associated genes *OsNPF6.1* (LOC_Os01g01360) and *OsNiR/PSR1* (LOC_Os01g25484) located in chromosome 1; *OsGS1;1* (LOC_Os02g50240) located in chromosome 2; *OsNPF2.4* (LOC_Os03g48180) located in chromosome 3; *OsNAC42* (LOC_Os09g32040) located in chromosome 9; *NRT1.1B* (LOC_Os10g40600) located in chromosome 10 and *TONDI* (LOC_Os12g43440) located in chromosome 12. Among them, two novel genes, *OsNPF6.1* and *OsNAC42*, were associated with enhanced NUE, effective panicle number and grain yield by increased nitrate uptake under low N supply. Using diverse rice germplasm panels collected from different eco-geographical regions, Liu et al. (2021) performed a GWAS on the tillering response to N and identified *OsTCP19* located in chromosome 6 as a modulator of this tillering response through its transcriptional activity to regulate the tiller-promoting gene *DWARF AND LOW-TILLERING (DLT)*. Interestingly, an allele of *OsTCP19* is linked with a high tillering response to N is predominant in wild rice populations. However, it has been lost in modern cultivars, suggesting that it might have contributed to geographical adaptation in rice.

5. HTP-assisted breeding of NUE

Two forward genetics approaches, viz., QTL mapping and GWAS, are normally used to explore the natural genetic variations present in crops. The linkage map constructed based on variations present in two parental lines contrast for the trait of interest along with their segregating populations is QTL mapping. The GWAS is the marker-trait association study that captured the phenotypic variation present in the diverse genotypes. HTP, combined with functional genomic studies, ML and GeS approach, provides a new era for breeders to capture nature's dynamic and speed up crop improvement (Bevan et al., 2017; Yang et al., 2020).

5.1. HTP and genetic mapping

HTP on important agronomic and NUE-related traits has been successfully utilized for GWAS and QTL mapping in plants such as *Arabidopsis* (Awlia et al., 2021), rice (Yang et al., 2014; Campbell et al., 2015; Al-Tamimi et al., 2016; Tanger et al., 2017; Guo et al., 2018; Wu et al., 2019), maize (Muraya et al., 2017; Zhang et al., 2017; Prado et al., 2018; Wang et al., 2019), cotton (Li et al., 2020), sorghum (Zhou et al., 2019; Miao et al. 2020a,b; Mantilla-Perez et al., 2020), barley (Honsdorf et al., 2014) and wheat (Lyra et al., 2020). Interestingly, HTP was utilized for identifying the QTL associated with temporal and developmental dynamics in *Arabidopsis* (Moore et al., 2013; Awlia et al., 2021). Moore et al. (2013) reported the time-dependent QTLs associated with root gravitropism on chromosomes 1, 3, and 4 using image-based root phenotypic traits from two independent populations of 162 recombinant inbred lines (RILs) and 92 near-isogenic lines (NILs). This group has demonstrated the utility of computer vision techniques and the power of statistical genetic analysis for characterizing the genetic architectures of developmental timing. To understand the dynamic and temporal physiological responses of plants under salt stress, Awlia et al. (2021) conducted GWAS of a diverse panel of 191 *Arabidopsis* accessions and identified 50 novel phenotypic traits highly dynamic for eight-time points from one hour to seven days after salt stress induction. The results showed that 770 loci were identified specific to salt stress, in which two loci were associated with maximum quantum yield and quantum yield measured in the light-adapted state (Fv/Fm).

Yang et al. (2014) developed a high-throughput rice phenotyping facility (HRPF) incorporating both HTP and GWAS to estimate diverse phenotypic traits across 529 rice accessions. The genetic basis of 15 important agronomic traits was identified, and GWAS dissected 141 associated loci, and the majority (25) of them are well-known genes. Recently, HTP has allowed the dissection of the genetic components of time-based salt stress responses in rice. Campbell et al. (2015) combined image processing software – Image Harvest, to process RGB images and fluorescence (FLUO) images and performed a longitudinal marker-trait association study to understand the genetic architecture of salinity-induced growth responses in 378 diverse rice genotypes. The results showed that, salinity-induced growth response as the ratio of PSA in salt-treated plants over the control at early state associated with genomic region on chromosome 3. A longitudinal model has been used and successfully identified 55 QTL with 115 highly significant SNPs associated with the dynamic response to salt stress in rice. Combining HTP and GWAS across 553 genotypes divided into two rice diversity panels (297 indica and 257 aus varieties), Al-Tamimi et al. (2016) identified a previously undetected locus affecting transpiration use efficiency Os11g07230 (encoding a receptor kinase) and Os11g07240 (encoding a serine/threonine-protein kinase BRI1-like 2 precursor) on chromosome 11, providing understandings into the early responses of rice to salinity. In both studies reported by Campbell et al. (2015) and Al-Tamimi et al. (2016), the genetic dynamics at different time points identified several time-specific QTL that moderately influence the final phenotype. However, these observed QTLs can only be detected at a specific time point but not in other stages of crops, suggesting the contribution of developmental stage-specific molecular mechanisms.

Tanger et al. (2017) performed field HTP phenomobile combined with genetic mapping from 1,751 F7 RILs derived from biparental of IR64 and Aswina. The overlapped QTL link with HTP phenotyping traits (canopy height, CT depression, normalized reflectance bands: NDVI, NDRE, Chla) and ground truth measurement (flowering time, height, biomass, grain yield, harvest index) has maximized genetic signal and improved the genomic prediction. Integrating GWAS, linkage mapping and HRPF to study drought tolerance across 507 diverse rice varieties, Guo et al. (2018) identified a total of 470 loci with 443 loci (94 %) identified using 51 image-based traits (i-traits). Out of which, 437 loci (93 %) were found to be co-localized with already-known QTLs associated with drought tolerance. Whereas GWAS analysis of 313 loci (66.6 %) was reproducibly validated in different years. To increase, the number and estimation accuracy of novel traits, Wu et al. (2019) studied a collection of 234 rice accessions at nine-time points using an RGB imaging system, and a total of 739 traits were non-destructively extracted with 74 morphological traits (tiller, shoot, biomass). GWAS study of these traits together identified 402 significantly associated loci, with 182 and 332 loci associated with traits measured independently using micro-CT and RGB, respectively.

6. Conclusions

Rice is a major cereal grown worldwide and consumed more than half of the population. Low NUE in rice demands the excessive application of N fertilizers that negatively impact farmer income and ecosystem. Breeding rice with higher NUE is critical for sustainable development to produce more gains per unit of fertilizer applied. To improve the NUE in rice, screening accessions and identifying superior donor genotypes is an essential step in a breeding program. NUE is a complex quantitative trait. HTP utilizes non-destructive sensors enabling spatiotemporal phenotyping of a large population set with high precision and accuracy in crop improvement. A uniform, controlled environment is critical for dissecting the component traits of highly complex NUE. Recent advances in crop phenomics and genomics provide powerful tools for quantifying appropriate traits and discovering QTLs associated with N deficit stress in plants. GWAS of natural genetic variation is considered a potential tool that accelerates the discovery of key genes and alleles associated with the NUE traits. Therefore, combining high-throughput phenotyping, next-generation genotyping data for genome-wide association studies and linkage mapping will help dissect the genetic architecture and improvement of NUE in rice.

CRedit authorship contribution statement

Nguyen Trung Duc: Writing – original draft, Resources, Formal analysis, Data curation. **Amooru Harika:** Writing – review & editing, Resources, Formal analysis. **Dhandapani Raju:** Writing – review & editing, Supervision, Software, Resources, Project administration, Funding acquisition, Data curation, Conceptualization. **Sudhir Kumar:** Writing – review & editing, Visualization, Supervision, Methodology. **Renu Pandey:** Writing – review & editing, Visualization, Supervision. **Ranjith Kumar Ellur:** Writing – review & editing, Supervision. **Gopala Krishnan S:** Writing – review & editing, Supervision. **Elangovan Allimuthu:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Biswabiplab Singh:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation. **Ayyagari Ramlal:** Writing – review & editing, Visualization, Supervision, Formal analysis. **Ambika Rajendran:** Writing – review & editing, Visualization, Supervision. **Ranjeet Ranjan Kumar:** Writing – review & editing, Supervision. **Madan Pal Singh:** Writing – review & editing, Supervision. **Rabi Narayan Sahoo:** Writing – review & editing, Supervision. **Viswanathan Chinnusamy:** Writing – review & editing, Supervision.

Declaration of competing interest

Authors have no competing interest to declare.

Acknowledgments

This work was funded by the National Agricultural Science Fund, ICAR, New Delhi, grant nos. NFBSFARA/Phen-2015, NASF/Phen6005/2016–17 and ICAR-Indian Agricultural Research Institute, New Delhi, grant no. CRSCARISIL20144047279. Student research grant provided to NTD by the National Agricultural Higher Education Project-Center for Advancement in Agricultural Science & Technology (NAHEP-CAAST) is fully acknowledged.

Funding statement

Not applicable.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

Data availability

No data was used for the research described in the article.

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