



Physiological traits and expression profile of genes associated with nitrogen and phosphorous use efficiency in wheat

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Abstract

Background Nitrogen (N) and phosphorous (P) play a very important role in the growth and development of wheat as well as major constituents of biological membranes. To meet the plant's nutritional demand these nutrients are applied in the form of fertilizers. But the plant can utilize only half of the applied fertilizer whereas the rest is lost through surface runoff, leaching and volatilization. Thus, to overcome the N/P loss we need to elucidate the molecular mechanism behind the N/P uptake.

Methods In our study, we used DBW16 (low NUE), and WH147 (high NUE) wheat genotypes under different doses of N, whereas HD2967 (low PUE) and WH1100 (high PUE) genotypes were studied under different doses of P. To check the effect of different doses of N/P, the physiological parameters like total chlorophyll content, net photosynthetic rate, N/P content, and N/PUE of these genotypes were calculated. In addition, gene expression of various genes involved in N uptake, utilization, and acquisition such as Nitrite reductase (*NiR*), Nitrate transporter 1/Peptide transporter family (*NPF2.4/2.5*), Nitrate transporter (*NRT1*) and NIN Like Protein (*NLP*) and induced phosphate starvation (*IPS*), Phosphate Transporter (*PHT1.7*) and Phosphate 2 (*PHO2*) acquisition was studied by quantitative real-time PCR.

Results Statistical analysis revealed a lower percent reduction in TCC, NPR, and N/P content in N/P efficient wheat genotypes (WH147 & WH1100). A significant increase in relative fold expression of genes under low N/P concentration was observed in N/P efficient genotypes as compared to N/P deficient genotypes.

Conclusion Significant differences in physiological data and gene expression among N/P efficient and deficient wheat genotypes could be useful for future improvement of N/P use efficiency.

Keywords Nitrogen use efficiency (NUE) · Phosphorous use efficiency (PUE) · qRT-PCR

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Abbreviations

Ct	Threshold cycle
DMSO	Dimethyl sulphoxide
GPP	Grain yield per plant
IPS	induced phosphate starvation
NiR	Nitrate reductase
NLP	NIN Like protein
NO ³⁻	Nitrate
NPF	Nitrate transporter 1/Peptide transporter family
NPR	Net photosynthetic rate
NRT	Nitrate transporter
NUE	Nitrogen use efficiency
PHO2	Phosphate 2
PHT	Phosphate Transporter
PUE	Phosphorous use efficiency
qRT-PCR	Quantitative real time PCR
TCC	Total chlorophyll content

Introduction

Bread wheat (*Triticum aestivum* L.) is one of the most important cereal crops and used to feed a large part of the world's population for many centuries. Besides a significant part of the world's population diet, it also serves as an important source of energy, protein, vitamins, and other beneficial compounds, not only for humans but also as animal feed [1]. It will be more challenging for the ever-growing population by 2050 to meet the nutritional demand and food availability. It is because of a decrease in cultivated land area, change in climate, and reduction in soil nutrients. Nitrogen (N) and phosphorus (P) are the most vital macronutrients required for the growth and development of wheat. Nitrogen plays important role in nucleic acid metabolism, chlorophyll as well as in protein synthesis and results in an increase in cell surface area, photosynthetic activity, and ultimately the growth and yield of plants [2].

After nitrogen, phosphorous (P) is the second most important plant nutrient that affects the growth, quality, and yield production of crops. Motsara [3] reported the presence of low (< 12.5 kg/ha) to medium (12.5–22.5 kg/ha) P in 80% of the soil samples collected in India. Therefore, to accommodate the needs of N and P in soil, these are replenished by the use of fertilizers. During the last 40 years for doubling global food production, an increase of 7- and 3.5-fold in consumption of nitrogen and phosphorous fertilizers, respectively was observed. It is observed that further doubling of global food production during the next four decades would require a 3.15-fold and 2.5-fold increase in the total N and P application, respectively. However, crop plants absorb only 30–50% of applied nitrogen fertilizers and less than 20% of applied P fertilizers, and the rest is lost through surface runoff and leaching. Phosphorous obtained from non-renewable sources such as mines and rocks are immobilized in soil due to chemical fixation and microbial activity [4]. Nitrogen/phosphorous loss results in the depletion of their use efficiency which is measured as grain yield produced as a function of N/P available to that plant [5]. However, the increased use of N and P fertilizers increased the expenses of farmers as well as harming the environment through the process of eutrophication and leaching [6].

Many studies have shown the effect of N and P fertilizers on wheat growth, yield, and physiological parameters such as total chlorophyll content (TCC) and net photosynthetic rate (NPR) [7–10]. To adapt to the limiting nutrient conditions, plants have evolved several complicated physiological and biochemical responses like inhibition of primary root elongation, increase in density and growth of lateral roots, decrease in CO₂ assimilation rate, and photosynthesis [11–13]. The

molecular mechanisms regulating these responses have involved the genes which help in the uptake, transport, localization, and accumulation of these limiting nutrients [14–17]. The candidate gene-associated study (CGAS) was conducted for NUE-related traits on a panel of 286 wheat varieties [18]. The *TaNRT2.1-6B* overexpression enhanced the nitrate influx and root growth of wheat, whereas the opposite effects were observed in gene silence lines. Kumar et al. [19] identified the N and P responsive 38 candidate genes which play a crucial role in N/PUE in wheat. Out of these 38 genes, miRNA for 10 candidate genes were also identified which regulate the transport and acquisition of N and P and showed significant differences under N starvation conditions.

To minimize the cost and application of N and P fertilizers, we need to focus on the approaches which enhance the N and P use efficiency of wheat genotypes and also need to understand the molecular mechanisms behind the uptake, transport, and utilization of N and P. By keeping this in view, the expression of these N and P responsive genes were studied using quantitative real-time PCR (qRT-PCR) in wheat genotypes DBW16 and WH147 under different concentrations of N and in HD2967 and WH1100 under different concentrations of P. Apart from this, the effects of different doses of N and P were also studied on physiological traits in the screen house.

Materials and methods

Plant materials and growth conditions

The study was carried out in the screen house of the Department of Molecular Biology & Biotechnology, CCS Haryana Agricultural University, Hisar. Wheat genotypes used for the study were selected based on their use efficiency for N and P as reported in our previous study [13]. Seeds of four wheat genotypes- WH147 (high NUE), DBW16 (low NUE), HD2967 (low PUE) and WH1100 (high PUE) were sown in a completely randomized block design (CRD) in triplicates in earthen pots. Nitrogen was applied in 3 different treatments as CaNO₃·4H₂O; 0.18 g low (LN), 0.36 g optimum (ON), and 0.74 g high nitrogen (HN). Phosphorous was given in the form of KH₂PO₄ as 0.07 g low (LP), 0.15 g optimum (OP), and 0.30 g high (HP), and other macro and micronutrients were supplied in the form of Hoagland solution. Fresh leaf samples were collected after 7 days of heading for physiological traits like chlorophyll content and net photosynthetic rate. The experimental material and

treatment conditions used in this study have given in Supplementary Table 1.

Moreover, to study the involvement of these genes in the uptake, transport, and assimilation of N and P, seeds of four wheat genotypes- WH147, DBW16, HD2967, and WH1100 were washed with double-distilled water (ddH₂O) and then surface sterilized with 0.1% mercuric chloride (HgCl₂) for 4–5 min. Seeds were thoroughly washed 4–5 times with ddH₂O to remove the traces of HgCl₂ and germinated over Petri plates. At two leaves stage, seedlings were transferred to hydroponics having Hoagland solution. Two sets of hydroponics were arranged each for N and P treatments. For 15 days, an adequate supply of N (2 mM) and P (0.2 mM) was given to plants. Solutions were replaced after every five days. On the 16th day, half of the plants were transferred separately to N (200 µm) and P (20 µm) deficient medium to impose the N and P stress and the other half were used as control (N, 2mM and P, 0.2 Mm). After 48 h of imposing stress, samples of roots and shoots were collected separately for RNA extraction and stored at -80°C for further use. An illustration of the methodology followed in this study is given in Fig. 1.

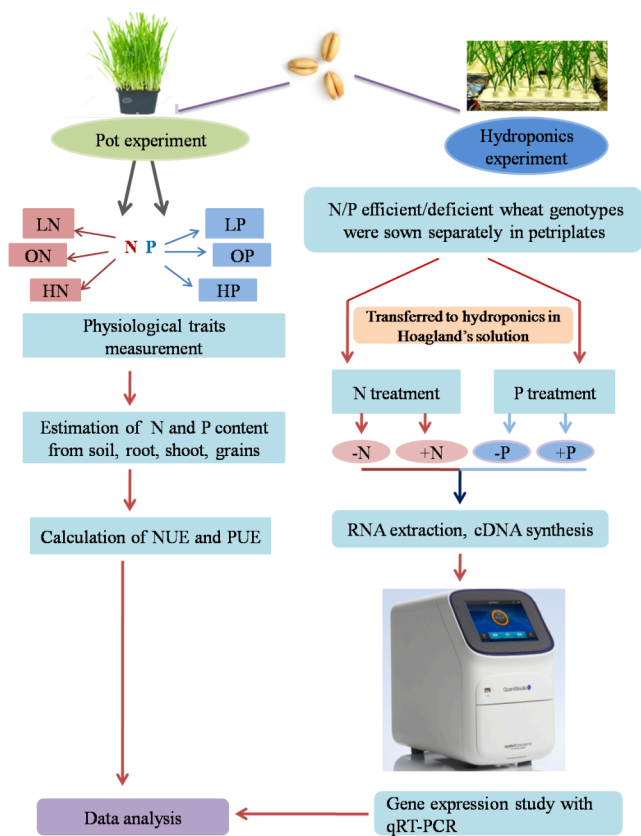


Fig. 1 Flow chart of the methodology used in the present investigation

Physiological traits measurement

Total chlorophyll content (TCC) was estimated from fresh leaves after 7 days of heading using the modified Arnon method [20]. Fully expanded leaf tissue of 50 mg was finely chopped and DMSO (Dimethyl sulphoxide) was added in a glass tube followed by incubations at 65°C for 1 h. After cooling at room temperature, observations were measured on a spectrophotometer at wavelengths 454, 654, and 663 nm.

The net photosynthetic rate (NPR) was determined on a fully expanded flag leaf after 7 days of heading using a portable IRGA (Infrared gas analyzer) ADC BioScientific LCi-SD System Serial No.33,956.

For NUE, N content was measured in roots, shoots, spikes and grains with the method of Lindner, 1944 and in soil by Kjeldahl's method [21]. Dried plant materials were digested individually in a mixture of sulphuric acid (H₂SO₄) and hydrogen peroxide (H₂O₂) in a closed microwave digestion system and diluted up to 25 ml with distilled water. One ml of digested plant material was taken to which 2 ml of Nessler's reagent was added, and orange color intensity was read at 440 nm. After harvesting, soil samples from each pot were also collected and dried in shade at room temperature for N analysis by Kjeldahl's method [21].

For PUE, P content was measured by the Vanadomolybdophosphoric yellow color method [22]. One ml of digested plant material was taken in a volumetric flask and 2–3 drops of 2,4 di-nitrophenol indicators were added to it. Ammonia solution was added to the volumetric flask till yellow color appeared, after that 6 N HCl (dropwise) was added till the yellow color disappeared. Vanadomolybdate solution (5 ml) was added to the flask and diluted to 25 ml with distilled water. Yellow color intensity was measured at a wavelength of 440 nm. Phosphorous analysis of soil samples was done by Olsen's Method [23].

RNA extraction and gene expression analysis

RNA was extracted from 100 mg of roots and shoots of selected wheat genotypes in the Maxwell RNA Extraction automatic machine (Promega). The single-stranded cDNA was synthesized from 1 µg RNA using Revert Aid 1st strand cDNA Synthesis Kit (Thermo Scientific). A total of 8 genes were selected having the role in uptake, transport and localization of N, and P in wheat, and their specific primers were designed using the PrimerQuest™ tool of integrated DNA Technologies (IDT) given in Supplementary Table 2. Their expression was studied under different N and P doses using an Applied Biosystems (Quant Studio 6 Flex) instrument. The wheat *actin* gene (accession no. AB181991.1) was used as an internal control. The expression of target genes and

endogenous gene (*actin*) was performed in triplicates for each sample. The following protocol was used to perform the qRT-PCR: UDG-activation at 50°C for 2 min, denaturation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 15 sec, annealing at 54–60°C for 15 sec, and extension at 72°C for 1 min. The average threshold cycle (Ct) comparative method was used to quantify the expression of the target genes [24]. The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

Data for various parameters were subjected to analysis of variance (ANOVA) using the STAR 2.0.1 version (Statistical Tool for Agriculture Research) according to a Completely Randomized Block Design (CRD). The data were presented as the mean \pm SE ($n=3$) and differentiated using the least significant difference (LSD) t-test at a 5% significance level ($p \leq 0.05$).

Results

Grain yield and physiological traits under different doses of nitrogen

Grain yield per plant (GPP), total chlorophyll content (TCC), and net photosynthetic rate (NPR) were observed higher under high N (HN) whereas NUE was observed higher under low N (LN) (Fig. 2a).

A significant increase in GPP was observed with an increase in N dose. Grain yield per plant (GPP) was

reduced to 40% in DBW16 and 11% in WH147 under LN, whereas under HN, GPP was increased by 30% in DBW16 and 32.25% in WH147. Total chlorophyll content (TCC) was found positively correlated with increasing nitrogen dose. Under LN conditions, TCC was reduced to 22.6% in DBW16 and 30% in WH147, whereas under HN conditions, an increase of 17% and 21% was observed in DBW16 and WH147, respectively.

The net photosynthetic rate was found to be reduced by 51% under LN in DBW16 and 38% in WH147. Whereas under HN, an increase of 10% was observed in DBW16 and a 22.7% increase was observed in WH147.

Nitrogen use efficiency (NUE) was found to be increased by 6.8% in DBW16 and 26% in WH147 under LN condition, whereas under HN conditions, NUE was reduced to 32 and 40% in DBW16 and WH147, respectively (Fig. 2b). Among all the three N doses, nitrogen content was found lowest under LN dose followed by ON and, HN in all the tissues of DBW16 as compared to WH147. While comparing all the plant tissues, N content was found higher in grains followed by shoot and root (Supplementary Table 3).

Grain yield and physiological traits under different doses of phosphorous

Physiological traits like total chlorophyll content (TCC) and net photosynthetic rate (NPR) were found lower under LP (low P) as compared to HP (high P) (Fig. 3a). Under LP, GPP was found to be reduced by 36% in HD2967 and 7.98% in WH1100 whereas, under HP, 11% increment was observed in HD2967 and 6.5% in WH1100. Under LP, TCC was reduced to 18 and 38% in HD2967 and WH1100,

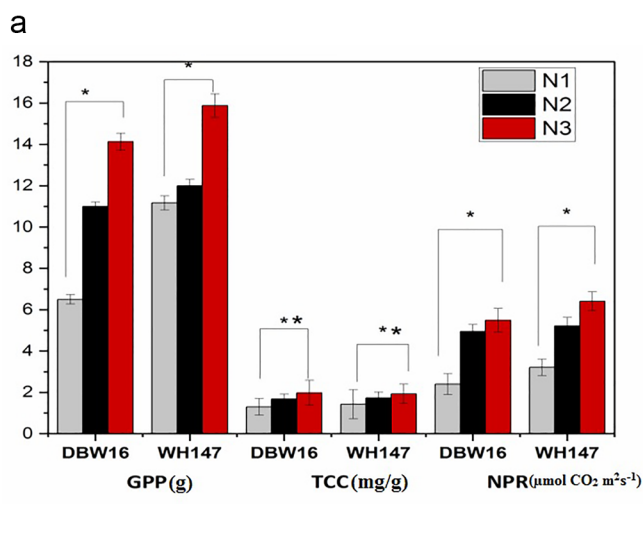
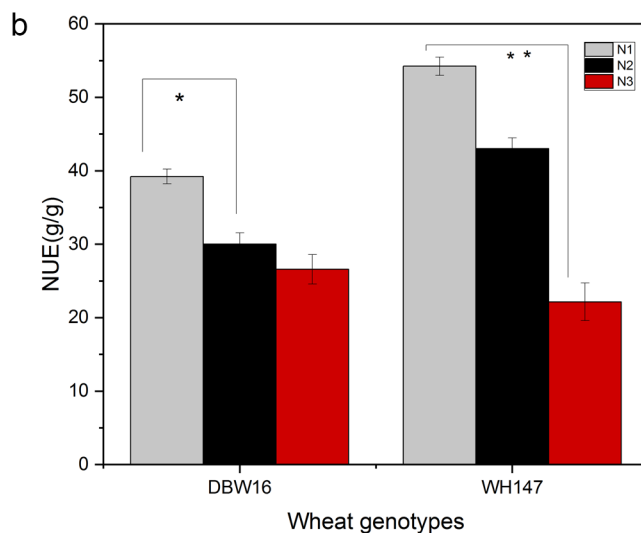


Fig. 2 Different physiological traits under low (N1), optimum (N2) and high (N3) doses of nitrogen in DBW16 and WH147 wheat genotypes. Values were the mean \pm S.E of three replications, * and ** indicate that the difference between the means was significant at P, 0.05 and P,



0.01, respectively. **(A)** Grain yield per plant (GPP), total chlorophyll content (TCC), and net photosynthetic rate (NPR) **(B)** Nitrogen use efficiency (NUE).

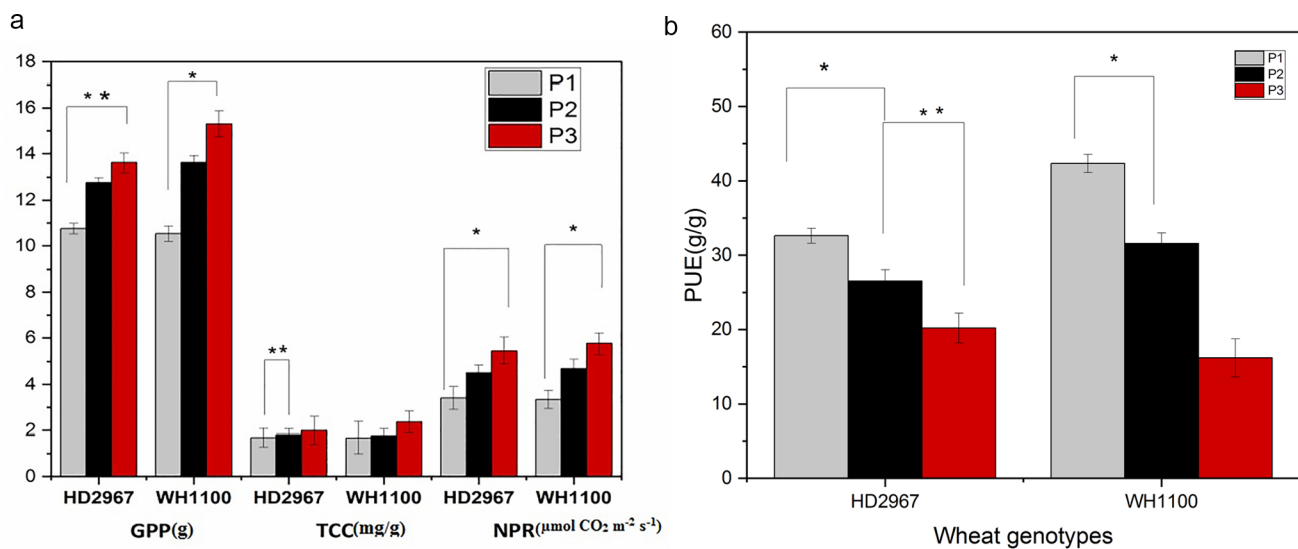


Fig. 3 Different physiological traits under low (P1), optimum (P2) and high (P3) doses of phosphorous in HD2967 and WH1100 wheat genotypes. Values were the mean \pm S.E of three replications, * and ** indicate that the difference between the means was significant at P,

respectively. Under HP, an increment of 34 and 48% was observed in HD2967 and WH 1100, respectively. Under LP, NPR was reduced to 24 and 21.28% in HD2967 and WH1100, respectively. An increment of 28% was observed in HD2967 and 22.55% in WH1100 under HP.

Under LP, phosphorous use efficiency (PUE) was increased by 22% in HD2967 and 34% in WH1100. Under HP, PUE was decreased by 23 and 48% in WH1100 and HD2967, respectively (Fig. 3b). Phosphorous content was found lowest under LP in all the tissues of HD2967 as compared to WH1100 under OP and HP conditions (Supplementary Table 4).

Relative gene expression analysis of N and P-responsive genes

For the relative expression analysis, the most commonly occurring genes which help in the uptake, transport and utilization of N and P, were selected. Relative expression of N-responsive genes was studied in two wheat genotypes- WH147 (high NUE) and DBW16 (low NUE) under two different N concentrations- 2 mM (adequate) and 200 μM (deficient/stress). Nitrogen-responsive genes used for expression analysis were Nitrite reductase (*NiR*), Nitrate transporter 1/Peptide transporter family (*NPF2.4/2.5*), Nitrate transporter (*NRT1*) and NIN Like Protein (*NLP*) which plays important role in uptake, transport, and distribution of N in different parts of wheat.

Whereas, relative expression of P-responsive genes was studied in two different wheat genotypes- WH1100 (high PUE) and HD2967 (low PUE) under two different P

0.05 and P, 0.01, respectively. (A) Grain yield per plant (GPP), total chlorophyll content (TCC), and net photosynthetic rate (NPR) (B) Phosphorous use efficiency (PUE).

concentrations- 0.2 mM (adequate) and 20 μM (deficient). P-responsive genes used for relative expression in this study were induced phosphate starvation (*IPS*), Phosphate Transporter (*PHT1.7*) and Phosphate 2 (*PHO2*).

Expression analysis of genes associated with uptake and transport of N

Nitrate transporter 1/Peptide transporter families (*NPF2.4/2.5*), and Nitrate transporter (*NRT1*) are low-affinity transporters which help in the uptake of nitrate. The relative fold expression of *NPF2.4/2.5* and *NRT1* was found higher in the genotype with more NUE (WH147) as compared to the genotype with low NUE (DBW16). Expression of *NPF2.4/2.5* was found 1-fold in DBW16 shoots and increased up to 1.81-fold in WH147 shoots. Expression of *NPF2.4/2.5* was found 1.55-fold in roots of DBW16 and increased up to 2.42-fold in roots of WH147 (Fig. 4a). *NRT1* expression was found 2.27-fold and 2.0-fold in roots of WH147 and DBW16, respectively. It was 0.55-fold in shoots of DBW16 and increased up to 1.3-fold in WH147 shoots (Fig. 4b).

Expression analysis of *NLP* gene

The relative fold expression of the *NLP2* gene was found higher in roots and shoots of WH147 (high NUE) than DBW16 (low NUE). It was 0.54-fold in shoots of DBW16 and increased up to 0.75-fold in WH147 shoots. Whereas, its expression was 1.00-fold in the roots of DBW16 and increased up to 1.16-fold in WH147 roots (Fig. 4c).

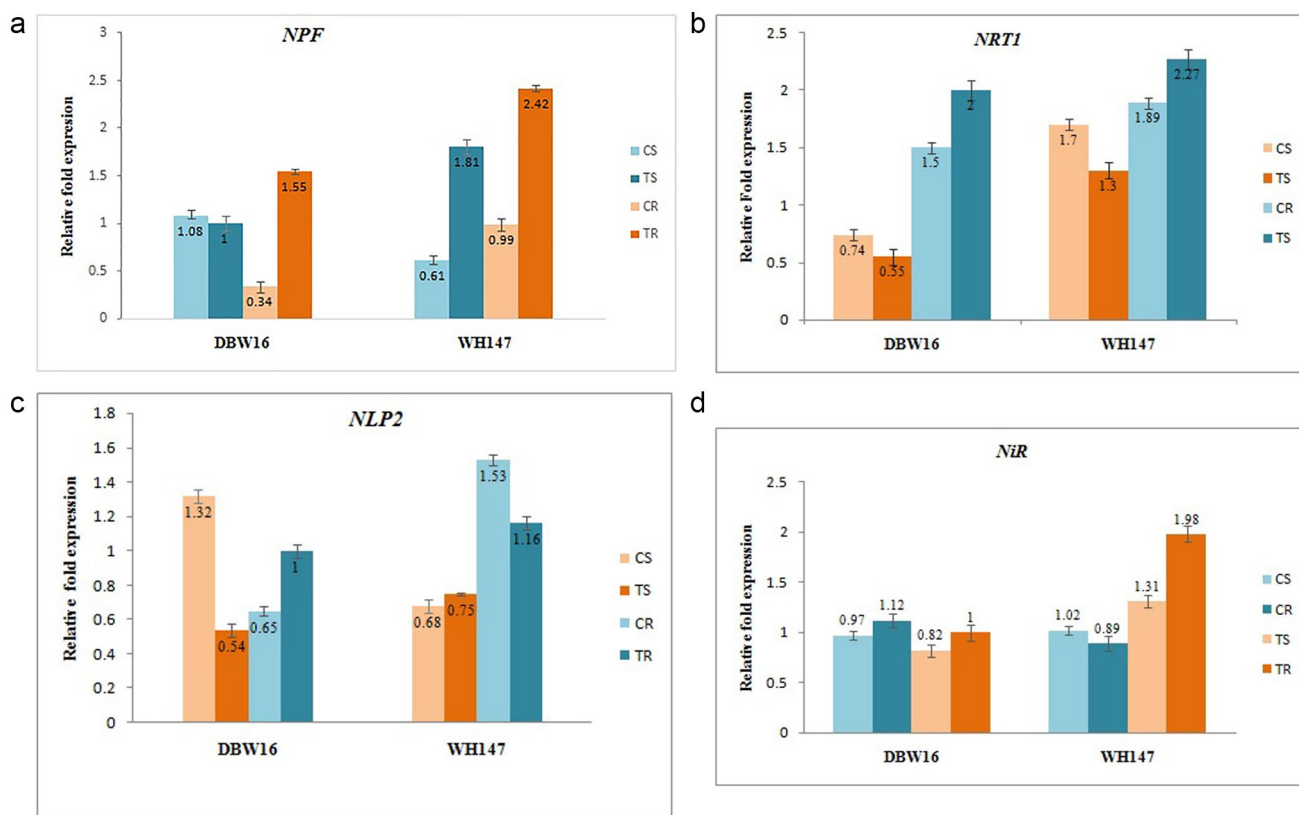


Fig. 4 Expression analysis of genes in roots and shoots of DBW16 and WH147 wheat genotypes under nitrogen stress. Values were shown as Mean \pm SE (n=3). *TaActin* was used as an internal control for data

normalization. **(A)** NITRATE TRANSPORTER 1 (*NRT1*)/PEPTIDE TRANSPORTER (*PTR*) family (*NPF 2.4/2.5*) **(B)** Nitrate transporter (*NRT1*) **(C)** Nitrate reductase (*NiR*) **(D)** NIN-like protein (*NLP*).

Expression analysis of genes associated with reduction and assimilation of N

The relative expression of *NiR* under two different N conditions in the root and shoot of DBW16 and WH147 was studied. The relative fold expression of *NiR* was found higher in the roots and shoots of WH147 as compared to DBW16. It was 1.12-fold in shoots of DBW16 and increased up to 1.31-fold in shoots of WH147. While *NiR* expression was 1-fold in DBW16 roots and increased up to 1.98-fold in WH147 roots (Fig. 4d).

Expression analysis of P-responsive genes

The relative fold expression of *PHT1.7* member of the Phosphate transporter gene family was studied in roots and shoots of two wheat genotypes- HD2967 (low PUE) and WH1100 (High PUE). It was found higher in shoots of both genotypes as compared to roots. Its expression was 3-fold in shoots of HD2967 and increased up to 3.51 in shoots of

WH1100. Whereas, its expression was 1.7-fold in the roots of HD2967 and increased up to 3.03-fold in WH1100 roots (Fig. 5a).

The relative fold expression of the *PHO2* gene was found higher in the roots of both the HD2967 and WH1100 as compared to shoots. Its expression was 0.92-fold in shoots of HD2967 and increased up to 1.18-fold in shoots of WH1100, While, its expression was observed 0.66-fold in roots of HD2967 and increased up to 1.52-fold in roots of WH1100 (Fig. 5b).

The relative fold expression of *IPS* was found higher in the roots of both the HD2967 and WH1100 as compared to shoots. Its expression was only 0.03-fold in shoots of HD2967 and increased up to 1.75 in shoots of WH1100. Whereas, its expression was observed 0.23-fold in roots of HD2967 and increased up to 15-fold WH1100 roots (Fig. 5c).

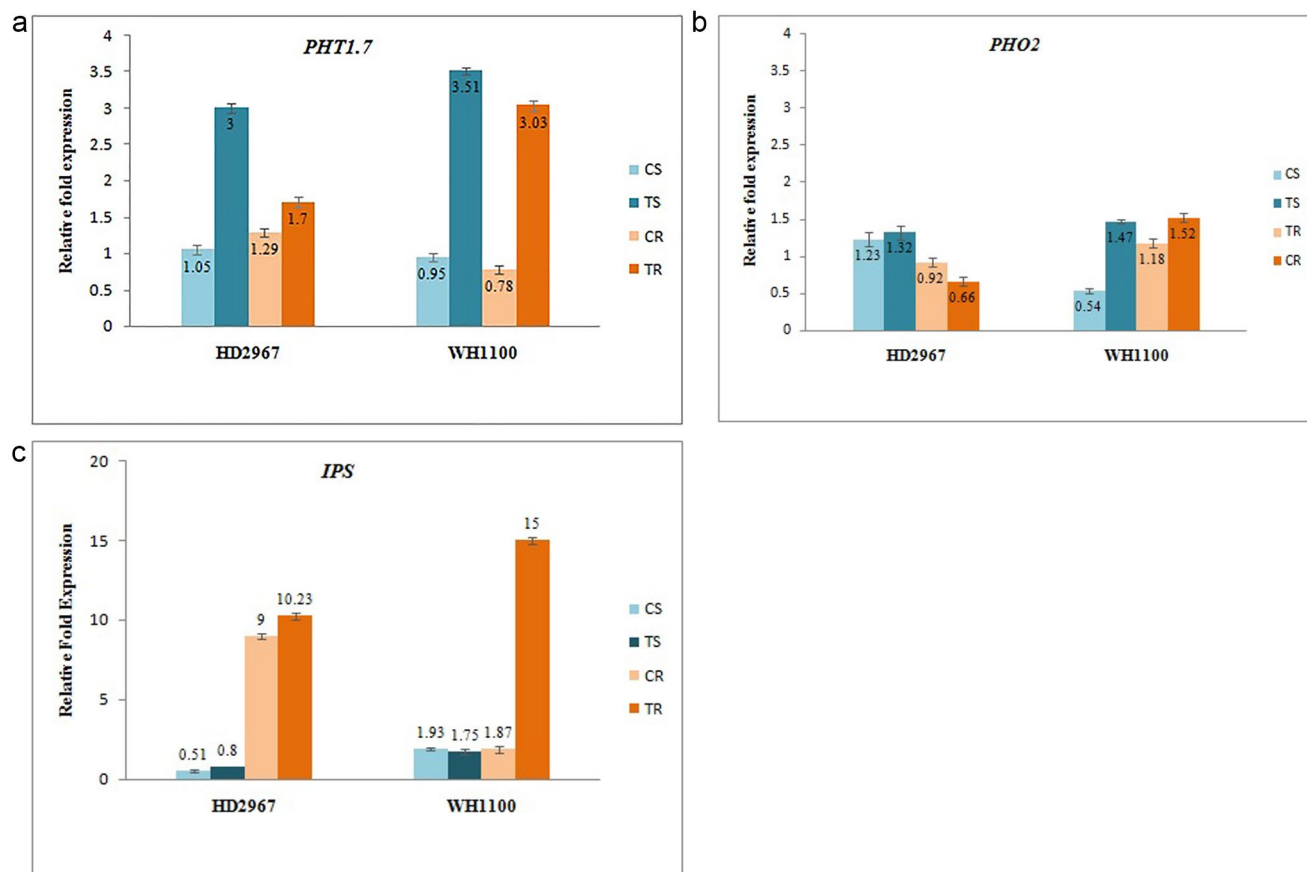


Fig. 5 Expression analysis of genes in roots and shoots of HD2967 and WH1100 wheat genotypes under phosphorous stress. Values were shown as Mean \pm SE (n=3). *TaActin* was used as internal control for

data normalization (A) Phosphate transporter 1.7 (*PHT1.7*) (B) Phosphate 2 (*PHO2*) (C) Induced phosphate starvation (*IPS*).

Discussion

Wheat production needs to be increased with the exponentially growing population to meet their nutritional demand [25]. Most cereal crops including wheat are deficient in nitrogen and phosphorous macronutrients. The deficiency of these nutrients in the soil ultimately reduces crop yield [26]. Farmers oversupplied the cereal crops with N and P fertilizers by aiming too high productivity. It has been reported that half of these applied fertilizers are lost through leaching and the surface runoff which results in several environmental concerns such as contamination of groundwater and eutrophication of water bodies [27]. Thus, the loss of these nutrients leads to a decrease in their use efficiencies and ultimately, the yield [28]. To minimize the loss of these nutrients, it is necessary to understand the molecular mechanisms of uptake, transport, and utilization of N and P in wheat. Therefore, we conducted this study to check the physiological and molecular response of selected wheat genotypes under N and P-stressed conditions.

Effect of different nitrogen and phosphorous dose on yield and physiological traits

In wheat, N absorption enhances from emergence to tillage, therefore, to attain the estimated wheat production, an appropriate N dose should be applied at the proper growth stage. A remarkable increase in grain yield and straw is also achieved through the application of N fertilizers in wheat [25]. N and P concentrations are significantly correlated with the content of chlorophyll in wheat plants and leaves [29–31]. The present research showed that total chlorophyll content was reduced under LN and LP conditions. The net photosynthetic rate (NPR) of genotypes under low doses of N and P was observed low as compared to the optimum dose. A deficiency of N and P diminished the electron transport from photosystem II (PSII) to photosystem I (PSI). Moreover, P deficiency may restrict photosynthesis by influencing the carbon translocation from the chloroplasts and preventing the efflux of triose phosphates from chloroplasts. Hence, the carbohydrate accumulation causes feedback inhibition of the electron transport chain which leads to a reduction in NPR and TCC [32–34]. The usage of high

nitrogen dose i.e., above 151 kg ha^{-1} , when the field is scarce with N, is not favourable and will increase the chances of lodging and disease in plants [25].

Grain yield per plant (GPP) was found higher under the optimum dose than the LN and LP doses. Similar results were reported by Abedi et al [35], higher grain yield was obtained under treatment with 240 kg N ha^{-1} (Optimum N) as compared to 120 kg N ha^{-1} (lower N), and 360 kg N ha^{-1} (Higher N). Tan et al. [36] observed the wheat production and performance under three fertilizers N rates (135, 180, and 225 kg N ha^{-1}) with two irrigation quotas and concluded that wheat production was higher under the optimum dose of fertilizer N rate at 180 kg N ha^{-1} .

Nitrogen and Phosphorous use efficiency

Nitrogen/Phosphorous use efficiency (N/PUE) is a measure of grain yield produced as a function of the N and P available to soil (Moll et al., 1982). N/P use efficiency was observed higher under a low application rate of N/P and reduced with an increase in the dose of N/P. Maximum NUE (24.8%) was found under the low N rate (135 Kg ha^{-1}), while minimum (22%) was found under a high N rate (225 Kg ha^{-1}) [36]. Similar results of maximum NUE under LN and minimum NUE under HN were observed in the present study. The highest NUE (21.72 kg kg^{-1}) was recorded in Control (T1) while the lowest NUE was recorded under T7 N treatment (13.39 kg kg^{-1}) [37]. Sandana and Pinochet [38] reported 72 g/g and 39 g/g PUE at different P application rates – 100 kg ha^{-1} and 250 kg ha^{-1} respectively. In the present study, PUE was observed highest under low doses in comparison with optimum and high doses. In another study, under adequate supply of P, PUE ranged from 300.9 to $1467.8 \text{ mg}^2 \text{ grain yield } \mu\text{g}^{-1}$, whereas under P stressed condition, it ranged from 369.0 to $1143.5 \text{ mg}^2 \text{ grain yield } \mu\text{g}^{-1}$ [39].

Expression analysis of genes under N stress

To adapt to the limiting nutrient conditions, plants have evolved several complex physiological and biochemical responses. The molecular mechanisms, regulating these responses, involve genes which help in the uptake, transport, localization, and accumulation of these limiting nutrients [16, 17], [40, 41] (Fig. 6). Thus, there is a need to understand the molecular mechanism adapted under limiting N and P conditions, which will be helpful in the development of N and P use efficient wheat varieties. Therefore, we studied the expression analysis of N-responsive genes such as *NRT1*, *NPF2.4/2.5*, *NiR*, and *NLP* in two wheat genotypes DBW16 (low NUE) and WH147 (high NUE) under two different concentrations of N; 2 mM (adequate) and 200 μM (deficient) after 48 h exposure to N stress. Similarly, the

expression analysis of P-responsive genes such as *IPSI*, *PHO2* and *PHT1.7* were studied in HD2967 (low PUE) and WH1100 (high PUE) of two wheat genotypes under two different concentrations of P; 0.2 mM (adequate) and 20 μM (deficient) after 48 h of stress exposure.

Nitrate transporter- NPF (NRT1/PTR) is the low-affinity nitrate transporter and is involved in nitrate uptake by roots and its transport within the plant. The relative fold expression of *NPF2.4/2.5* was found higher in WH147 (high NUE) as compared to DBW16 (low NUE). It was 1.08-fold in DBW16 shoots and increased up to 1.81-fold in shoots of WH147. Whereas, its expression was 1.55-fold in the roots of DBW16 and increased up to 2.42-fold in the roots of WH147. Wang et al. [42] studied the expression of *NPF* genes under different N starvation conditions – 200 kg ha^{-1} (high) and no N (low) concentration in roots, and leaves. In roots, expression was found to be increased up to 3-fold whereas in leaves it increased up to 1.5-fold.

NRT1.1 functions as a dual-affinity transporter and is predominately expressed in the epidermis of younger root tips and its expression is inducible under nitrate (NO_3^-). This gene can sense external NO_3^- and activate the NO_3^- signaling pathway. The mutants of *nrt1.1* showed marked nitrate uptake defects in comparison to wild-type plants, in both high and low-affinity ranges [43]. In the present study, expression of *NRT1* was found higher in N efficient wheat genotype WH147 than DBW 16 which is N deficient wheat genotype. We studied the expression of this gene on the 2nd day of N exposure and found 2.27-fold expression in the roots of WH147 and 2.0-fold expression in DBW16. One another study [44] found the expression of the TaNRT1.1 gene dramatically increased at the 2nd and 4th days after N starvation of wheat seedlings. Its expression was higher in roots (2.5-fold) under the N starving condition as compared to the control condition (1.0-fold).

Jagadhesan et al. [45] studied the relative expression of the *NiR* gene involved in N assimilation under the three different N conditions in roots and shoots of four genotypes of rice Apo, IR83929-B-B-291-3-1-1 (IR-3-1-1), Nerica-L-42 (NL-42), and Pusa Basmati 1 (PB1). Among all the three N treatments, *NiR* expression was found higher in roots of NL-42 genotypes as compared to shoots, under treatment 1 (3.5-fold) followed by treatment 2 (3-fold). NL- 42 has the highest NUE among all the four genotypes at a low N rate. Expression of *NiR* gene in high NUE demonstrates the role of *NiR* genes in N signaling and assimilation of N. In the present study, expression of *NiR* was found higher in roots of WH147 (1.98-fold) as compared to DBW16 (1.00-fold),

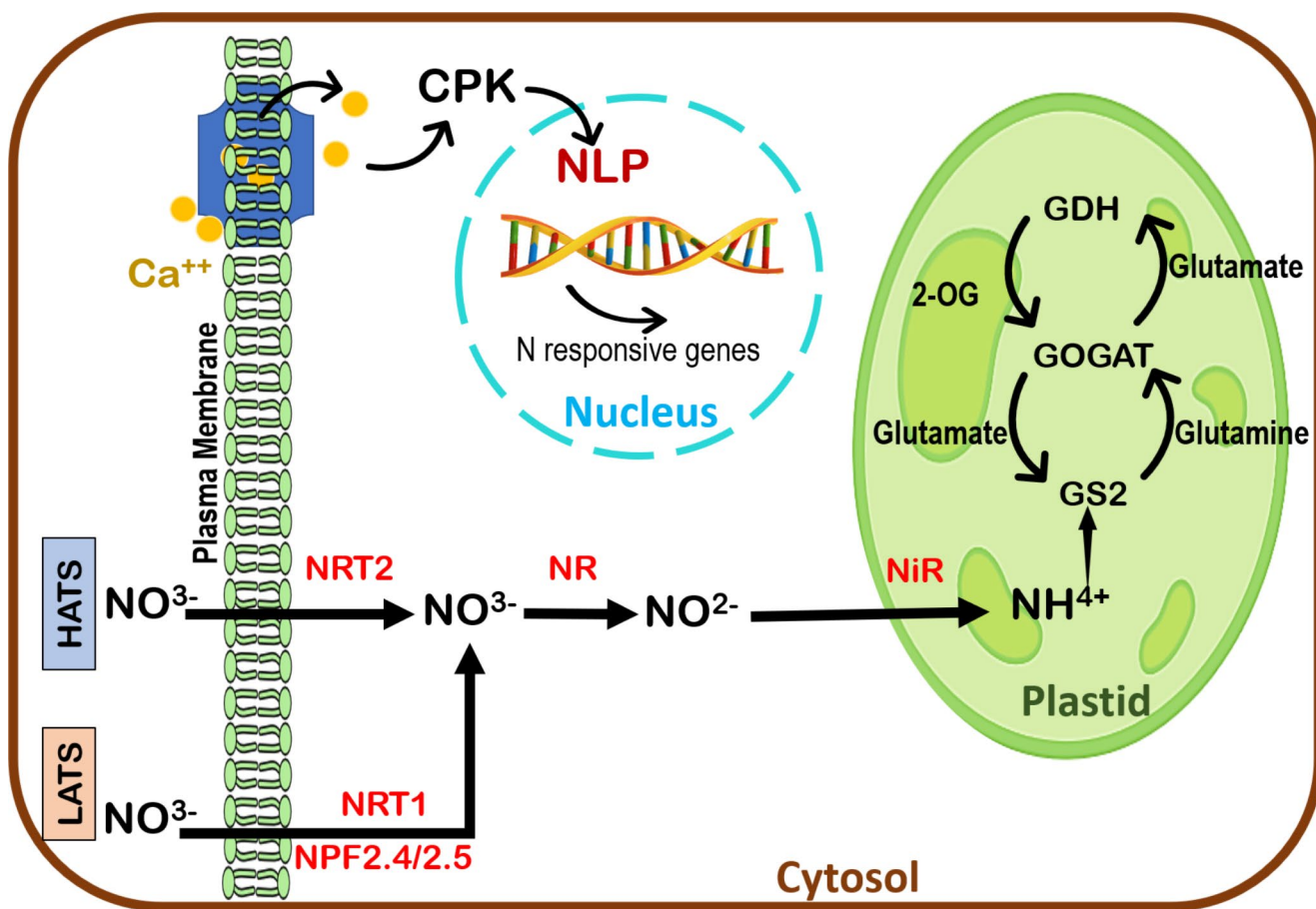


Fig. 6 Pathway shows the gene(s) involved in plant nitrogen uptake and metabolism in red. Plants uptake NO_3^- through roots, followed by nitrite reduction resulting in ammonium ions which are fixed into amino acids glutamine and glutamate. LATS (low-affinity transporter), HATS (high-affinity transporter), nitrate transporter 1 (*NRT1*), Nitrate

transporter 2 (*NRT2*), nitrite reductase (*NiR*), nitrate reductase (*NR*), glutamine synthetase (*GS*), glutamate synthase (*GOGAT*), glutamate dehydrogenase (*GDH*), NIN LIKE protein (*NLP*)

whereas in shoots it was reduced to 1.31-fold in WH147 and 0.82-fold in DBW16.

Several transcription factors (TFs) have proven to be involved in N use and major switches in plant regulatory networks. Several genes like *NRT1.1*, *NRT2.1*, and *NRT2.2* involved in the NO_3^- uptake and signalling pathways are regulated by members of the *NIN-like protein (NLP)* family of TFs, such as *NLP2*, *NLP6*, *NLP7*, and *NLP8*. In the present study, expression of the *NLP2* gene was found 1.16-fold in roots of WH147 and 1.0-fold in DBW16 roots, whereas in shoots it was reduced to 0.75-fold in WH147 and 0.54-fold in DBW16. Similar results were obtained in NL-42 rice genotypes (high NUE) by Jagdhesan et al [45]. The 18 *NLP* genes were identified in wheat by using in silico approach *NLP1*, *NLP7* and *NLP2* showed significant differences among all the identified *NLP* genes under N stress. Further validation of these genes by using qRT-PCR also showed a significant difference in their expression i.e *NLP 7* was

found up-regulated in roots and shoots of higher NUE wheat genotypes [46].

Expression analysis of genes under P stress

Under P starvation conditions, the *IPSI* signaling cascade plays a crucial role in the uptake and transport of inorganic phosphorous (Pi) (Fig. 7). Plants uptake phosphorous via the Pi transporter present in root epidermal and cortical cells. From cortical cells, it is uploaded to the xylem for further translocation to shoots and retranslocated to roots via the phloem. *IPSI*, phosphate transporter (PHT) plays important role in the acquisition, translocation and remobilization of Pi. In the present study, we studied the relative expression of *IPSI* under P-deficient conditions in roots and shoots of HD2967 (Low PUE) and WH1100 (High PUE). Relative expression was found higher in roots of WH1100 (15-fold) as compared to roots of HD2967 (10.23-fold). Meanwhile, in shoots, the expression was 1.75-fold in WH1100 and

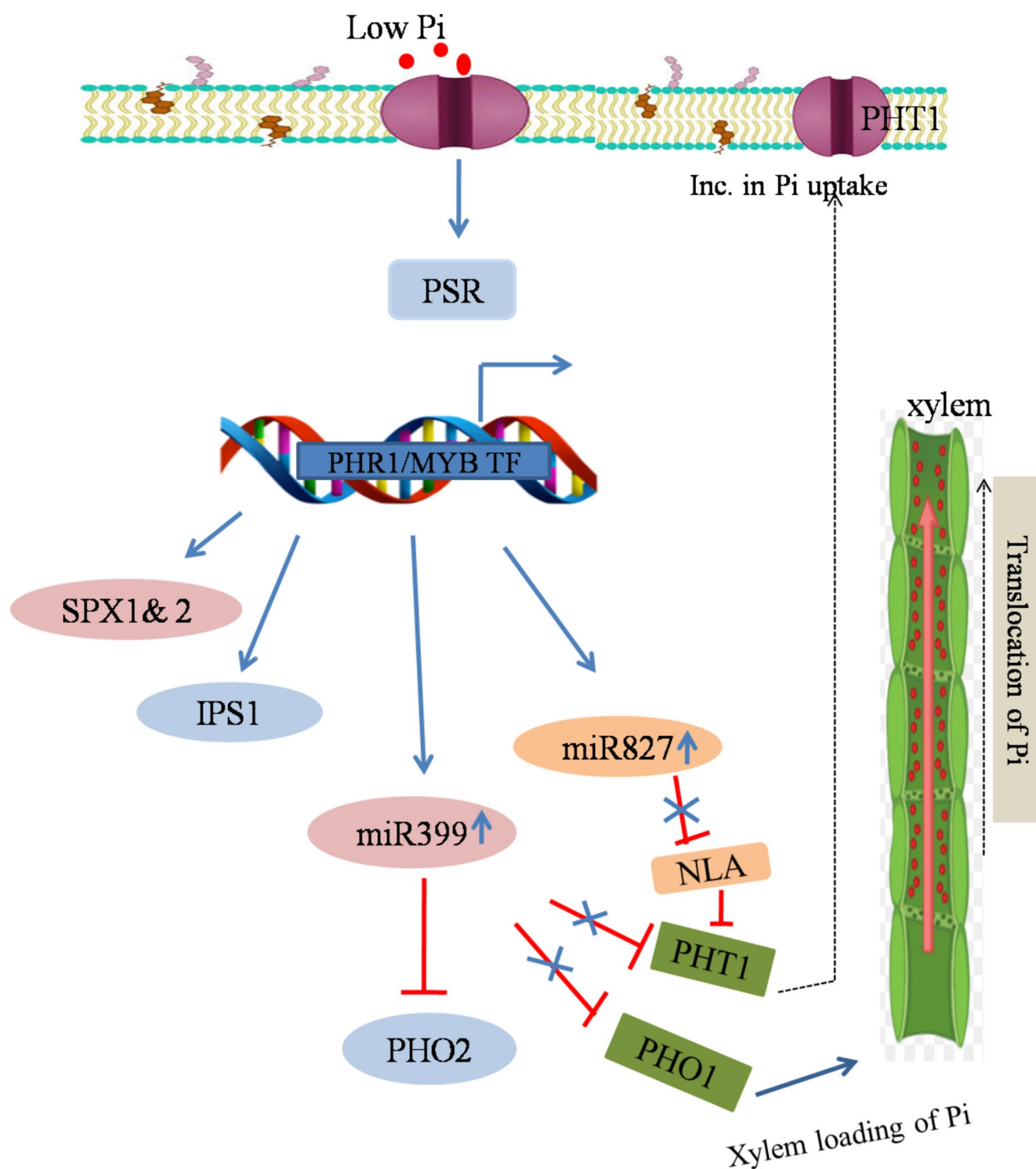


Fig. 7 Schematic representations of P stress response in plants. Pi starvation triggers the phosphorous starvation response (PSR), which leads to the induction of the Phosphate starvation regulator (PHR1), MYB TF that acted a role downstream of the phosphorus signal transduction pathway. *PHR1* is a negative regulator of P-starvation, it up-regulates the induced phosphate starvation (*IPS1*), *SPX1* and 2, *miR827*

and *miR399* expression. *miR399* and *miR827* represses phosphate 2 (*PHO2*) and Nitrogen limitation adaptation (*NLA*), respectively. *NLA* and *PHO2* repression results in the accumulation of phosphate transporter 1(*PHT1*) and *PHO1* which consequently increased Pi uptake and translocation from root to shoot

0.8-fold in HD2967. Oono et al. [47] (2013) studied the RNA Seq. and relative expression analysis of *IPSI* in wheat root and shoot under P starving conditions. The expression levels of *IPSI* increased 368-fold in roots and 17-fold in shoots as observed in qRT-PCR analysis and similar results were obtained using RNA-Seq analysis.

Under P starving conditions, *PHO2* negatively regulates the uptake and localization of Pi. Under low Pi conditions, *PHO2* degrades through the IPS-miR399-Pho2 signaling cascade. The expression of phosphate transporter genes (*Pht1;8* and *Pht1;9*) are activated due to reduced *PHO2* protein level, which leads to the facilitation of Pi uptake and transport to the shoot [40],[47, 48]. In the present study, we studied the expression of both *PHO2* and *PHT1.7* (phosphate transporter) in the root and shoot of HD2967 (Low PUE) and WH1100 (High PUE) under P-deficient conditions. Relative expression of *PHT1.7* was found higher in shoots of WH1100 (3.51-fold) as compared to HD2967 shoots (3.00-fold) whereas, in WH1100 roots, expression reduced to 3.03 fold and 1.7 fold in HD2967 roots. The relative expression of *PHO2* was observed higher in WH1100 roots (1.52-fold) as compared to HD2967 roots (0.66-fold). In shoots, expression reduced to 1.18-fold in WH1100 and 0.92-fold in HD2967. Ouyang et al. [49] studied the expression of *PHO2* under low P conditions and found 4.0-fold and 2.0-fold higher expression in roots and shoots, respectively.

Conclusion

This study characterized the physiological and molecular responses of selected wheat genotypes under nitrogen and phosphorous stress conditions. Physiological parameters and yield were reduced under low N and P treatments. Furthermore, significant differential expression of nitrate uptake genes such as *NRT1*, *NPF2.4/2.5* gene was found approx. 2-fold in roots of WH147 as compared to DBW16 and showed their role in uptake and metabolism and thus an increase in NUE of WH147. Under P stress, *PHT1.7* expression was found 3-fold higher in WH1100 roots which were observed just double from the HD2967 roots (1.7-fold) A significant difference in expression of these genes in selected wheat genotypes confirmed the effective and organized signal transduction network of these genes under nitrogen and phosphorous stress, which could be useful for improving N/P use efficiency of wheat.

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Author contributions UK conceived the idea and designed the experiment. VS and PS carried out the experiments. YS, PB, and KPS analysed the data. UK, VS and YS drafting and editing the manuscript. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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