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Development and characterization of nitrogen and phosphorus use efficiency responsive genic and miRNA derived SSR markers in wheat

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Among all the nutrients, nitrogen (N) and phosphorous (P) are the most limiting factors reducing wheat production and productivity world-wide. These macronutrients are directly applied to soil in the form of fertilizers. However, only 30–40% of these applied fertilizers are utilized by crop plants, while the rest is lost through volatilization, leaching, and surface run off. Therefore, to overcome the deficiency of N and P, it becomes necessary to improve their use efficiency. Marker-assisted selection (MAS) combined with traditional plant breeding approaches is considered best to improve the N and P use efficiency (N/PUE) of wheat varieties. In this study, we developed and evaluated a total of 98 simple sequence repeat (SSR) markers including 66 microRNAs and 32 gene-specific SSRs on a panel of 10 (N and P efficient/deficient) wheat genotypes. Out of these, 35 SSRs were found polymorphic and have been used for the study of genetic diversity and population differentiation. A set of two SSRs, namely miR171a and miR167a were found candidate markers able to discriminate contrasting genotypes for N/PUE, respectively. Therefore, these two markers could be used as functional markers for characterization of wheat germplasm for N and P use efficiency. Target genes of these miRNAs were found to be highly associated with biological processes (24 GO terms) as compared to molecular function and cellular component and shows differential expression under various P starving conditions and abiotic stresses.

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INTRODUCTION

Wheat is the most important cereal crop in the world. Most of the world's human population depends on cereal crops including wheat for their food and nutritional demand. Hexaploid wheat (2n = 6× = 42; AABBDD genomes), commonly known as bread wheat, originated from the combination of 3 interlinked diploid genomes having repetitive sequences accounting for approximately 80% of the total genome (~17 GB) (Venske et al. 2019). Wheat is in global demand and highly consumed in the industry due to its ability to make distinctive food products such as bread, biscuits, cakes, pasta, noodles, and grain alcohols, to name a few (https://www.britannica.com/plant/wheat). It is also consumed in daily diet as an important source of energy, iron, vitamin B, and proteins (Shewry and Hey 2015, Kumar et al. 2016). In the next 50 years, it will be a challenging task to meet out the food and nutrition demand for ever-growing human population (Ladha et al. 2016). The challenge is being aggravated by a decrease in cultivated land area, changing climate, food habitats, and reduction of nutrients in the soils. Nutrients in the soil serve as important limiting factor and play a vital role in the growth and development of wheat crop (Mathpal et al. 2018). The elemental nitrogen (N) have crucial role in nucleic acid metabolism, chlorophyll formation, and protein synthesis, successively results in increase in cell surface area, photosynthesis, and ultimately the growth and yield of plant. The deficiency of nitrogen can disturb plant biological processes like photosynthesis and yield-related traits such as grain weight and grain number (Fischer et al. 2013). Similarly, another macronutrient "phosphorous (P)" also plays a principal role in a set of biological processes including nucleic acid synthesis, respiration, and energy production (ATP) (Rouached et al. 2010). Elser et al. (2000) reported that 39% N and 9% P by mass is present in nucleic acids. Previous studies also showed that deficiency of either N or P leads to reduction in plant growth, development, and yield (Liu et al. 2016; Bogard et al. 2010; Xu et al. 2007; Longnecker et al. 1993). Therefore, there is need to supply these macronutrients to the soil in the form of fertilizers (Kulcheski et al. 2015). More "N" uptake resulted in increased crop production because cereal seeds reserve 6% N in the form of proteins (Ladha et al. 2016). It is estimated that plants utilized only 30–40% of applied fertilizers while the rest are lost through the surface run off, leaching, volatilization and denitrification (Plett et al. 2018; Correll 1998; Hirel et al. 2011). Nitrogen can also escape

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in the form of gases like ammonia (NH_3) and nitrous oxide (NO), which in turn may result in eutrophication of water bodies (Kumar et al. 2017a). Along with harming the environment, it is also costlier for the farmers (Kumar et al. 2017a; Plett et al. 2018). The loss of both N and P results in depletion of their use efficiency by plants (Balyan et al. 2016). Therefore, to overcome these problems it is required to improve nitrogen/phosphorous use efficiency (N/PUE) of wheat, so that maximum amount of N and P may be available to the plants (Plett et al. 2018; Chiou et al. 2006). Various approaches like conventional breeding and molecular breeding approaches such as marker assisted selection (MAS), transgenic, and miRNA manipulations have been suggested to improve the N/PUE in wheat.

Several studies inculpated the significant role of microRNAs in plant growth, development, signaling pathways, biotic and abiotic stresses such as heat stress and nutrient starvation conditions (Sunkar et al. 2012). The microRNAs are small (21–23 nucleotides long), endogenous RNA molecules that regulate the gene expression by cleavage and post-transcriptional regulation in plants as well as in animals (Kim 2005). Numerous reports have shown the miRNAs involvement in crosstalk between N and P (Kumar et al. 2017a; Jones-Rhoades et al. 2006; Xu et al. 2011; Vance 2010). Expression of miRNAs can be up-regulated or down-regulated under nutrient-deficient conditions (Wang et al. 2021; Li et al. 2016; Hou et al. 2020). Earlier studies have demonstrated the involvement of miRNAs in remobilization, uptake, and production of antioxidant scavengers under nutrient-deficient condition (Fischer et al. 2013; Paul et al. 2015; Zeng et al. 2014). The miRNAs can modify the growth and development of roots and flowers under both N and P stress (Nguyen et al. 2015; Zuluaga and Sonnante 2019; Rouached et al. 2010).

Molecular markers help in refinement of conventional breeding efficiency by selecting those traits which are tightly linked to marker, phylogenetic analysis, and characterization of crop germplasm (Gupta et al. 1999; Kumar et al. 2021). Different types of molecular markers have been developed and are available for crop improvement programs (Gupta et al. 2013a, 2013b; Sihag et al. 2021; Tyagi et al. 2019, 2021; Kumar et al. 2021). Among these, simple sequence repeats (SSRs) or microsatellites are co-dominant, multi-allelic, highly reproducible in nature, and present all around the genome of wheat in coding, non-coding, and un-translated region (UTR) (Gupta and Varshney 2000; Mir et al. 2013; Mir and Varshney 2012; Kumar et al. 2021). Due to these characteristics, SSRs proved ideal for plant molecular analysis and population genetics analysis (Mir et al. 2013; Mir and Varshney 2012; Gupta et al. 2012). For genetic mapping of wheat, >4000 of SSRs have already been developed, and significantly more can be generated because already developed SSRs are present throughout the genome at a frequency of only 29.73 Mb (Gupta and Varshney 2000).

Previous studies have reported the SSRs presence in pre-miRNA sequences of different plant species like Arabidopsis, rice, and wheat in response to different abiotic stresses (Mehta et al. 2021; Lawson and Zhang. 2006; Kumar et al. 2017b). Singh et al. (2018) have developed 65 salt-responsive gene-based SSRs in wheat and validated them on 60 wheat genotypes for genetic diversity analysis. Mehta et al. (2021) designed 53 salt-responsive genes and miRNA-based SSRs in wheat and screened them over 54 wheat genotypes for genetic diversity analysis for salt tolerance. These studies proved the role of miRNA-derived SSRs and gene specific SSRs in improving the performance of wheat under different abiotic stresses (Sihag et al. 2021; Tyagi et al. 2021; Hou et al. 2020; Ahmed et al. 2020). Zhang et al. (2019) analyzed the regulation of miRNA under high temperature in maize. Kumar et al. (2018a) performed a bioinformatics-based study to gain structural and functional insight into N and P responsive candidate gene. Yet, to the best of our knowledge, no report is available in wheat on miRNA-based SSRs in response to N and P efficiency/deficiency.

Therefore, in the present study we identified, developed, and validated the miRNA-based SSR markers along with genic SSRs in response to N and P use in wheat. The most important and diagnostic miRNA-derived SSR markers will prove useful in wheat molecular breeding programs aimed at enhancing N and P use efficiency. The markers will also prove useful in characterizing wheat germplasm for N and P use efficiency.

MATERIALS AND METHODS

Plant material and experiment design

A set of ten wheat genotypes/cultivars were judiciously selected for the present study based on earlier published literature on N/PUE (Table S1). To calculate the actual N/PUE of these genotypes, the experiment was carried out in earthen pots containing 5 kg sandy loam soil following a completely randomized block design (CRD) in triplicates. Seven seeds were sown in each pot and seedlings were thinned to five plants per pot. The amount of N and P was applied according to recommended dose-150 kg/ha and 60 kg/ha respectively (Kumar and Singh 2019). Nitrogen was applied in the form of $CaNO_3.4H_2O$ in two doses–0.18 gm (low) and 0.37 gm (optimum). Phosphorous was given in the form of KH_2PO_4 as 0.07 gm (low) and 0.15 gm (optimum). Other macro and micronutrients were supplied in the form of Hoagland's solution as per Hoagland and Arnon (1950). Soil analysis for N and P was done by Kjeldahl's (Subbiah and Asija 1956) and Olsen's methods (Olsen et al. 1954) respectively. Agronomic data like grain yield per plant, harvest index, biomass per plant were calculated for all the genotypes (Table S2 and S3). The N/PUE of each and every genotype was calculated following Moll et al. (1982).

DNA extraction and PCR amplification

A fresh leaf tissue (100 mg) was used for genomic DNA isolation from each genotype by using CTAB method (Cetyl tri-methyl ammonium bromide) (Doyle and Doyle 1987). The quality of genomic DNA was determined using agarose gel electrophoresis (0.8% w/v). Nanodrop spectrophotometer (Thermo Scientific) was used to perform DNA quantification. DNA was diluted to final concentration of 60 ng/µl for PCR and stored at −20 °C for further use.

For PCR amplification, a reaction mixture of 20 µl containing 2 µl DNA template (120 ng), 10 µl promega green master mix, 0.5 µl of each forward and reverse primer (0.2 µM) and 7.0 µl nuclease free water (NFW) was prepared. PCR program was optimized as: initial denaturation at 94 °C for 3 min, followed by 30 cycles of denaturation at 94 °C for 1.0 min, annealing temperature (50–58 °C) for 30 sec, extension at 72 °C for 30 sec and final extension at 72 °C for 7 min. The thermal cycler products were resolved on 10% poly-acrylamide gels at 120 V for 4 h (Maniatis et al. 1975). The gel was visualized in Vilber Fusion Solo S gel documentation system, after staining with ethidium bromide (EtBr). The scoring of genotypes was done on the basis of absence (0) and presence (1) of a specific allele.

Identification and designing of N and P responsive miRNAs SSRs

An extensive literature survey was conducted to collect the N and P stress-responsive miRNAs reported in the model plant Arabidopsis and certain cereals crops namely, wheat, maize, and rice. Premature miRNA (Pre - miRNA) sequences of N and P responsive miRNAs were obtained from miRBase22.0 repository (http://miRbase.org/) in FASTA format (Kozomara et al. 2019). In order to find out the 1000 bp (500 bp upstream and 500 bp downstream of the premature-miRNA sequence) long pre- miRNA sequence, BLASTn search was performed against wheat reference genome data publically available on Ensembl Plants database (https://plants.ensembl.org/) (Bolser et al. 2016). Results of BLASTn gave multiple hits, the sequence showing the alignment near to 100% on 0 e-value were chosen and utilized for SSRs mining followed by primer designing. Primers with di-, tri-, tetra-, penta-, and hexanucleotide repeat motifs were picked for primer designing by BatchPrimer3 v1.0 software (https://wheat.pw.usda.gov/demos/Batch Primer3/0) (You et al. 2008). Flow chart showing the representation of the process used for miRNA SSRs identification, validation, and characterization in wheat is presented in Fig. 1.

Development of gene specific SSR markers

Candidate genes associated with signaling, transport, uptake, and assimilation of N and P were collected from Arabidopsis, rice, and brachypodium based on the literature survey. Full length genomic sequences of these genes were extracted from different primary and secondary databases such as NCBI (www.ncbi.nlm.nih.gov), TAIR

(https://www.arabidopsis.org/), and MSU (http://rice.plantbiology.msu.edu/) in FASTA format. BLASTn search was performed against wheat reference genome data available on Ensembl Plants database. The SSRs motifs were developed by using BatchPrimer3 v1.0.

Diversity analysis and population differentiation

The polymorphism information content (PIC) of each primer was calculated by using GeneCalc bioinformatics tool (Nagy et al. 2012). DARwin v6.0 software was used to calculate the dissimilarity matrix (Perrier and Jacquemoud-Collet 2006). This matrix was used for clustering of 10 wheat genotypes and for dendrogram preparation with unweighted pair group method with arithmetic mean (UPGMA) procedure by bootstrapping with 1000 permutations (Gascuel 1997). To categorize these 10 genotypes into distinct groups, principal component analysis (PCA) was also performed using DARwin v6.0.

Number of alleles per locus (Na), number of effective allele per locus (Ne), the Shannons information index (I), observed heterozygosity (Ho), expected heterozygosity (He) were calculated by GenAlEx6.5 (https://biology-assets.anu.edu.au/GenAlEx/) (Peakall and Smouse 2006). GenAlEx6.5 software was run for analysis of molecular variance (AMOVA) to test the genetic variance among and between the populations (Shen et al. 2017).

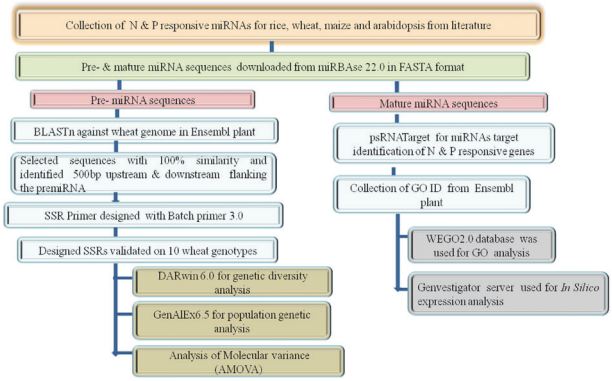

Fig. 1 Flow chart for illustrating the process of miRNA SSRs identification, validation, and characterization in wheat.

Gene ontology and miRNAs targets identification

Mature miRNA sequence retrieved from miRBase v22.0 were submitted as a query to find the miRNA targets in cDNA library of wheat followed by identification of miRNAs target gene in response to N and P from psRNA Target server (http://plantgrn.noble.org/psRNATarget/). WEGO database-Web Gene Ontology Annotation Plot (WEGO 2.0, https://biodb.swu.edu.cn/cgi-bin/wego/index.pl) was used for gene ontology (GO) studies of identified miRNA targets by retrieving GO ID of target genes from Ensembl Plants database.

In silico expression profiling of miRNA targeted genes

Expression profiling of predicted miRNA targets was performed using RNA seq experiments embedded in Genevestigator platform (https://genevestigator.com/gv/index.jsp) (Grennan 2006). A total of 40 miRNA target genes were used for expression profiling. Transcript IDs of these genes were utilized as query while performing the expression profiling under different environment stress conditions as well as nutrition starvation in wheat. Hierarchical clustering tool was used to generate heat map of expression profiling as earlier described by Kumar et al. (2018a).

RESULTS

Development of nitrogen and phosphorous responsive miRNA-SSRs in wheat

A total of 38 miRNA families and 8 candidate gene families responsive to N and P were identified on the basis of the literature survey. These miRNAs families consisting of 71 members, which were recognized from rice, Arabidopsis, maize, and wheat (Fig. 2A). During primer designing, no SSRs were found for five miRNAs

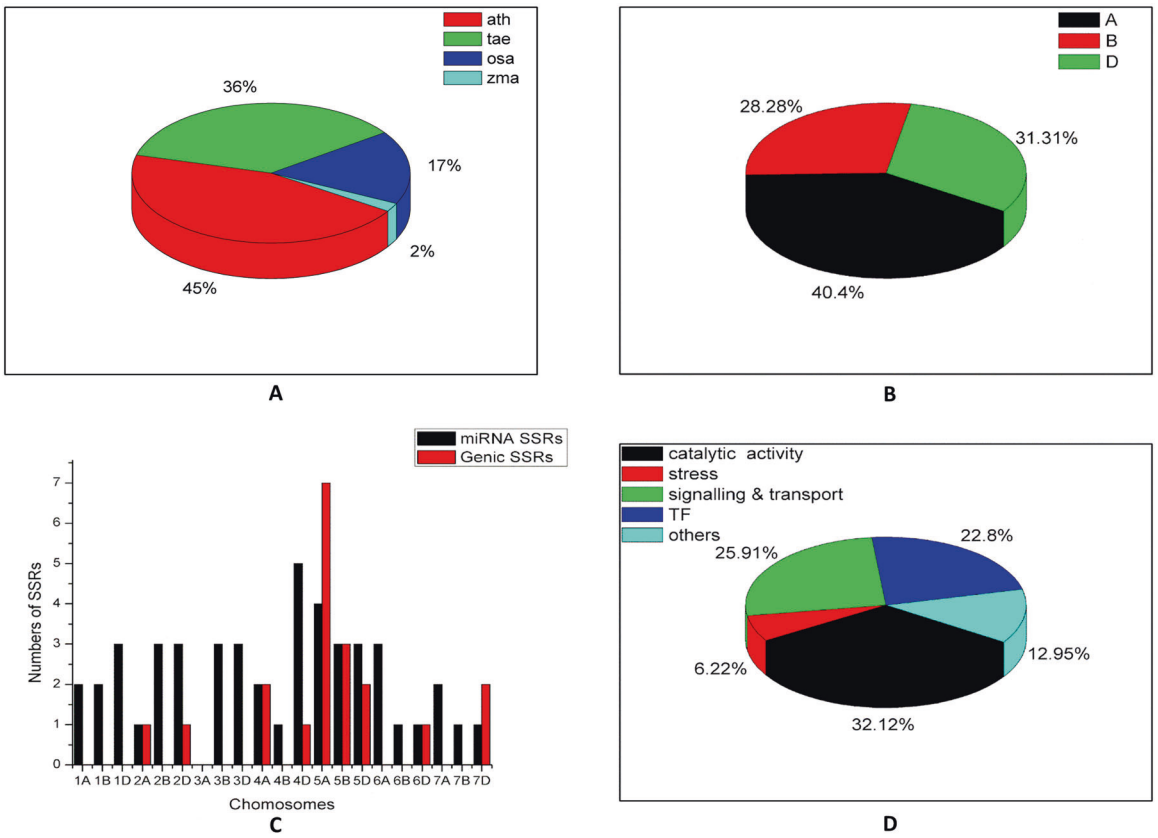

Fig. 2 Frequency and distribution of nitrogen and phosphorous responsive miRNA and gene-specific SSRs. A In various crops, **B** in subgenome of wheat, **C** in wheat chromosomes, and **D** on the basis of their role in different cellular activities.

i.e., miR1124, miR444, miR174, miR1117, miR1133, and one gene family i.e., TaPHT (TaPHT 1.6-4A, 1.6-4B, and 1.6-5D). Primers were designed by using BatchPrimer3 v1.0 and primers could be designed for 66 significant miRNA-SSRs and 32 gene-specific primer pairs. The repeat length in the SSRs varied from dinucleotide $(NN)_{24}$ repeat to hexanucleotide $(NNNNNN)_4$ repeat. Maximum numbers of miRNA genes were found to possess tetra-nucleotide repeats (45%) followed by tri- (40%), penta- (6%), hexa- (5%) and dinucleotide (2%) repeats. Maximum frequency of SSRs was found on wheat sub-genome A (40.4%) followed by sub-genome D (31.31%) and sub-genome B (28.28%) as shown in Fig. 2B. The N and P responsive SSRs were present on all 21 chromosomes of wheat except 3A. Maximum frequency (10%) of SSR loci was found on chromosome 5A, while the minimum frequency (2%) was found on 2A, 4B, 4D, 6A, and 6D (Fig. 2C). SSRs were also classified on the basis of their role in different activities/ functions: catalytic activity (32.12%), signaling transport (25.91%), Transcription factor (TF) in stress (6.22%), and others (12.95%) (Fig. 2D).

Validation of SSR markers and allelic diversity analysis

Total 98 SSR markers (66 miRNAs and 32 gene-specific SSRs) were selected for validation using 10 selected wheat genotypes for their N and P use efficiency (Table S4). Out of these 98 SSRs, 74 primer pair amplified the expected product size range and showed the clear and reproducible results. Among these, 35 SSRs showed polymorphism, out of which 24 were miRNA SSRs and 11 were gene-specific SSRs (Table 1). The miR167a detected maximum number of alleles i.e., 5 alleles. PIC analysis indicated that the PIC value ranged from 0.19- 0.99; miR165b and miR837 had lowest PIC value of 0.19, whereas as miR167a, miR846, and TaPHT1.54A had the highest PIC value of 0.99. Polymorphic markers "miR171a and miR167a" were found to be superior/best for genetic diversity analysis of wheat genotype for N/PUE, respectively. The banding patterns of miR171a and miR167a (Figs. 3 and 4) also showed an association of these markers with phenotypic data of N/ PUE. Therefore these markers will prove helpful in the selection of N/P efficient and deficient wheat genotypes in selection programs.

Genetic Diversity Analysis

The binary scoring data generated from N and P responsive polymorphic SSRs were used for diversity analysis using DARwin6 software which clustered these genotypes into 3 clusters- cluster I, II, and III. The wheat genotype DBW16 with low NUE was found to be more diverse than other genotypes and found in cluster I. Cluster II contains genotypes with low NUE except WH147 which have high NUE, whereas, Cluster III contains all the genotypes with high NUE except HD2967 and Choti Lerma which have low NUE. (Fig. 5). In case of P responsive SSRs, Cluster I and II comprised of genotypes with low PUE whereas cluster III has all the genotypes with high PUE except WH147 which have low PUE (Fig. 6). 2-D Principal component analysis (PCA) also separated the 10 wheat genotypes from each other in response to N and P as shown in Figs. 7 and 8, respectively.

Population differentiation

GenAlEx6.5 software was used to obtain genetic diversity parameters. Allelic data of N responsive SSRs have a total of 138 alleles in two populations. Among them, 78 alleles were found in population 1 and 60 alleles were found in population 2. Number of polymorphic loci in population 1 (93.75%), was observed to be higher than in population 2 (84.38%) (Table 2). Allelic data obtained from P responsive SSRs identified a total of 112 alleles in both populations. Among them, 58 alleles were found in population 1 and 54 were identified from population 2. Population 1 was found to be more polymorphic than population 2. Number of polymorphic loci for population 1 was observed to be 85.19%, whereas for population 2 it was 81.48% (Table 3).

To test the genetic variance among the population, AMOVA was performed (Tables 4 and 5). Total variance of 12% was observed among the population, whereas 88% variance was within the population under both N and P deficient/efficient conditions.

Target identification

psRNATarget server identified the 40 targeted genes of N and P responsive miRNAs (Table S5). The identified miRNA targets are well established for their critical role in a plethora of biological processes such as uptake, transport, signaling, and remobilization of nitrate and phosphate and in floral development of wheat. Signaling molecules regulated by these miRNAs targeted genes including serine/threonine-protein kinase, inositol- tetrakisphosphate 1-kinase, calcium-transporting ATPase, E3 ubiquitin-protein ligase and transcription factor like *NAC*, 6 A, *RNAC 1*, *EDR1*, *Auxin response factor* (ARF), R1R2R3-MYB protein, AP2, WALLS ARE THIN 1(WAT1) related protein, and Zinc finger protein. These miRNAs regulate the target genes by two mechanisms- cleavage and translational repression. miR775 and miR1123 target the phosphate transporter, whereas miR837, miR1128, miR1137, and miR156h target the genes involved in N cycle like ammonium transporter, nitrate reductase, glutamate receptor, respectively, whereas no target was found for miR160a. Many target genes such as laccase, auxin response factor, serine/threonine-protein kinase, and glycosyltransferase were found the target of more than two miRNAs. Target genes such as peroxidase (POD) and superoxide dismutase (SOD) were also found to have a vital role in antioxidant and secondary metabolite production under N and P stress. These metabolites help in scavenging of active reactive oxygen species (ROS) and protect the plant from photoinhibition under N and P deficient conditions (Poli et al. 2018).

Gene ontology analysis

GO studied by WEGO, categorized the miRNAs target gene's function into three groups- "molecular functions", "cellular components" and "biological processes". For molecular functions, 16 GO terms were found to be associated with ion binding, membrane transport, ligase, hydrolase, etc. The 15 cellular components GO terms were most abundant in cell part, intracellular organelle followed by intrinsic membrane part. Most of the target genes were linked with the biological process (24 GO terms) like response to abiotic stimuli, stresses, and primary metabolic processes (Fig. 9). Hence, GO analysis revealed the role of miRNAs target genes in uptake, transport, and signaling of N and P.

In silico expression analysis of miRNA target genes

In silico expression profiling of 40 miRNAs target genes was studied using RNA Seq data available under N and P deficient conditions in Genevestigator platform (Table S5). Expression of these miRNA-targeted genes was analyzed in six anatomical parts, five developmental stages under nutrient and abiotic stresses (Fig. 10). Most of the targeted genes were expressed moderately in six anatomical parts especially in roots, crown, and shoots (Fig. 10A). However, the expression potential percentage was higher in leaf followed by flag leaf and shoot. Among all the genes used in this study, genes encoding the ser/thr protein kinase and purple acid phosphatase were found to be highly expressed followed by phosphate transport protein, whereas gene for nitrate transporter was down regulated in almost all tissues. Expression profiling of all the 40 target genes was analyzed at various developmental stages like seedling growth, milk development, dough development, inflorescence emergence, and stem elongation. Target gene glutamate receptor and phosphate transport protein were up-regulated at inflorescence emergence, whereas SOD up-regulated at milk development stage (Fig. 10B). Stage-specific expression profiling of these genes infers their role in development and establishment of wheat.

Table 1. Details of 35 polymorphic miRNAs and gene specific SSR markers screened over a set of 10 wheat genotypes.

S. No.	miRNA name	SSRs motif repeat	Forward Primer sequence (5′–3′)	Reverse Primer sequence (5′–3′)	Tm (°C)	Amplicon size (bp)	No. of alleles	PIC
1	miR159a	(TTCT)4	CTCACCCCTCTATAAAACGAC	CTACATCTATGGGGCTAGGAG	50.2	80–100	2	0.84
2	miR159c	(CT)27	CTTTCCCTCGTGCTTGGAT	GCATAGTGATTTGATTTTCTTGTTAGC	52	150–400	3	0.95
3	miR159f	(CT)27	ACCTGTATAGGTTTTGCATGA	TTAGGTGCAGACTGAAAACAT	53	150–350	3	0.78
4	miR160a	(TCCT)3	TATATGCACCGATGGCACAG	CGAAACAGCTCCACAATTCA	54	150–300	3	0.67
5	miR160b	(TGGC)3	CCAGCTTGATGCAAAAGGTT	GGAAGGAAGGAAGGAAGGAA	54	150–400	3	0.67
6	miR164a	(GCCC)3	ACTGCACTGCACGTGTTCTT	TTGAAGACGCATACCTCGTG	54.3	150–400	3	0.97
7	miR 165b	(ATAC)10	CAACGGTGTGATTGTAAAAA	CGAAGTTTAATTTGGTTATGC	50	150–300	3	0.19
8	miR167a	(GGAC)5	AGGCCGCCATACAGAAGATA	TTCAGTCTCTCACACCAGCAA	55.6	150–400	5	0.99
9	miR167c	(AAAT)3	CCATCACTCCTCACTCATTCA	AAGCTCCTTACCGTGGTTCA	54	150–300	2	0.49
10	miR171a	(CGCC)3	GTCGGGGGACTTGAATGC	GGGAGAAGAGAGAGGGGAGA	52	150–400	3	0.77
11	miR172a	(AGAT)4	TATCTATGCATGAGCCAAGA	CGCCGCCTGTATTTACTAT	50.6	100–250	3	0.81
12	miR172c	(TTC)5	CCTCTCTTTGTCTTCATCCA	AAGAACCGACTGTGATCTGA	51.7	160–350	3	0.85
13	miR396	(TCC)6	GTGCCTTCTCTCTGCTCTATT	ATAATTCACATCACGGCAAG	52	120–240	3	0.85
14	miR396Db	(GTGC)5	GGTCGTGTGGAAGTTATATCG	GGCCGTATAAATAGAGCTTTG	51	150–500	4	0.92
15	miR396D	(GTGC)3	AAGTTATATCGGACCGTGTG	AGGAAGGGGTCGTATAAATAG	50.2	120–300	3	0.84
16	miR399d	(GAA)5	GTCTCTGGCGAGTGAGATAG	AATCTCCTTTGGCAGATAGAC	51.4	150–450	3	0.83
17	miR830	(AGG)4	AGTACGCCTTGATCTCCTCT	CTACGTTACCTTCCTCTCTCC	54	150–250	3	0.83
18	miR 837	(AAAGA)3	GACAACCGAATAAAATTGATG	GACACTTTGATAGCCACTGAG	49	150–170	2	0.19
19	miR846	(TATT)3	ACTAGTGGAAACATACACTTAAA	TTGAATAACCTGTTTCTGCTG	50.2	150–200	2	0.99
20	miR528	(CTG)5	GTACTGCCACCAACCTGA	AATTAAACACCCAAGCACAC	53	100–200	3	0.9
21	miR528a	(CTG)5	GTACTGCCACCAACCTGA	AATTAAACACCCAAGCACAC	53.2	140–600	3	0.88
22	miR 771 .2	(TCA)4	GCTAATCTGTTCACTGCTCAT	AATCTGAAGAGATCCCACCT	52.7	100–150	2	0.36
23	miR 1119	(AGCG)4	GGCTAAATAAAAGTGTTGTCG	GATCATAGGTGGAACTGACAC	49	150–350	3	0.87
24	miR 1123	(CCA)4	TACTAGCTGCAGCCGGAGAC	TACCGGTAATCGTCTTCGTA	52.7	180–220	2	0.19
25	OsENOD-93.2	(CCT)5	CTCCATCGTCATTTGCTG	AGTACAGCAGCAGGTCTCAT	53	150–300	3	0.75
26	OsENOD-93.3	(CTGCTC)3	ATCTTCATTTGCTGCTCTTG	CAGCATGTACAGGCACAG	51.5	100–250	3	0.75
27	OsENOD-93.4	(TAGGCA)4	CTGCTTCTGCTTCTACTGCT	GTCCACGTCTTCCTCCTC	54.8	150–200	2	0.92
28	AtSTP-13.2	(GAC)4	GTCTTCGTGCTCTTCTTCC	TAGGAGCAACACTTTTCAGAC	52.7	200-250	2	0.69
29	AtSTP-13.3	(CTGC)4	GTCTGAAAAGTGTTGCTCCTA	CGACTGAAAGTGTCTGCAC	52.3	130–250	2	0.56
30	TaGDH1-5A.1	(GGTTTG)3	GGGCATTAGAAAAGAGATAGC	GAAAAGTGGGGACGGATT	50.9	150	2	0.36
31	TaNT2.46D	(GCAG)3	GCAGAGAGTGGTAATTAAGAGG	GGATCAGATCAGAATATGCAC	50.6	150–250	3	0.72
32	TaNRT2.6 6A	(CATA)3	GTACATTCTTCTGCCTGTCTG	AGTAGGATACAACGCAGGAGT	51	150–300	3	0.75
33	TaPHT1.5 4A	(CGTG)3	GTAGCAACCAGGTGTGTAGAG	CTGTCCCTTTGAACTTATCG	53	150	2	0.99
34	TaPHT1.7 11- 4A	(CAC)4	GAGAAGCTCTCCAAGTTTAGG	GAAGATGTCCTTTTGTGTCAG	51.5	160	2	0.51
35	TaPHT1.7 11- 4D	(CAC)4	GAGAAGCTCTCCAAGTTTAGG	GAAGATGTCCTTTTGTGTCAG	51.4	150	2	0.69

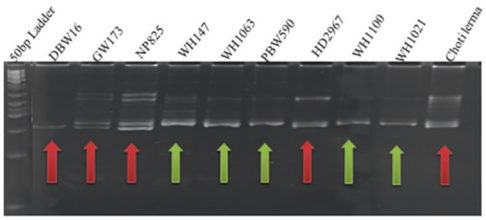

Fig. 3 10% Urea-PAGE profiling of miR171a marker screened over 10 wheat genotypes. Green and red arrow represent genotypes with low and high NUE respectively. Lane 1 depicts 50 bp DNA ladder and lane 2–11 represents 10 wheat genotypes.

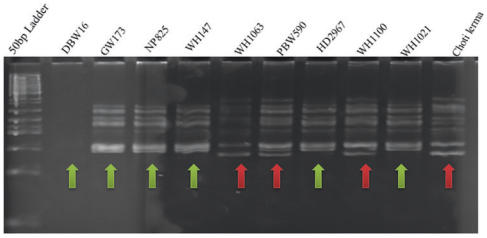

Fig. 4 10% Urea-PAGE profiling of miR167a marker screened over 10 wheat genotypes. Green and red arrow represent genotypes with low and high PUE respectively. Lane 1 depicts 50 bp DNA ladder and lane 2–11 represents 10 wheat genotypes.

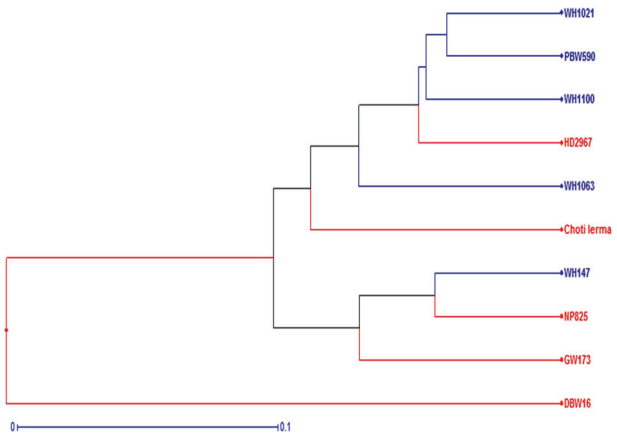

Fig. 5 Dendrogram showing clustering of 10 wheat genotypes on the basis of polymorphic N responsive miRNA and genic SSRs. Red and blue color indicates the N deficient and efficient wheat genotypes.

Under P starvation conditions, NAC transcription factor, zinc finger protein, and glycosyltransferase were found highly expressed (Fig. 10C). The expression profiling of these genes under low P implies their role in senescence and nutrient remobilization. Phenylalanine lyase and SOD were up-regulated under various stress levels. Flowering genes were down regulated under both nutrients and stress level. Therefore, expression profiling under stress responses also revealed the role of these genes in regulation of phosphorous metabolism under various environmental stresses.

Correlation among phenotypic and genotypic data

Efficient genotypes performed better as compared to deficient genotypes under both optimum and low condition of N and P. The phenotypic data of recorded traits showed clear differences between efficient and deficient genotypes under optimum and half dose of N and P (Tables 6 and 7). Two most promising/

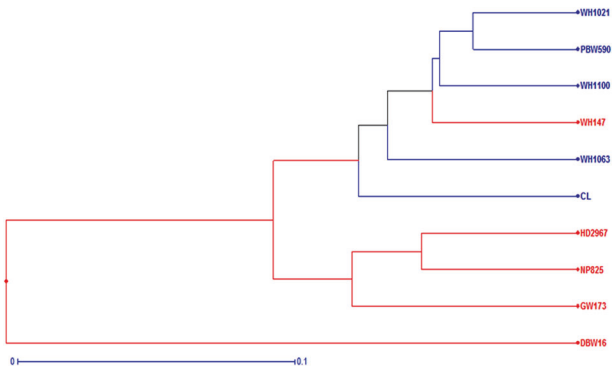

Fig. 6 Dendrogram showing clustering of 10 wheat genotypes on the basis of polymorphic P responsive miRNA and genic SSRs. Red and blue color indicates the P deficient and efficient wheat genotypes.

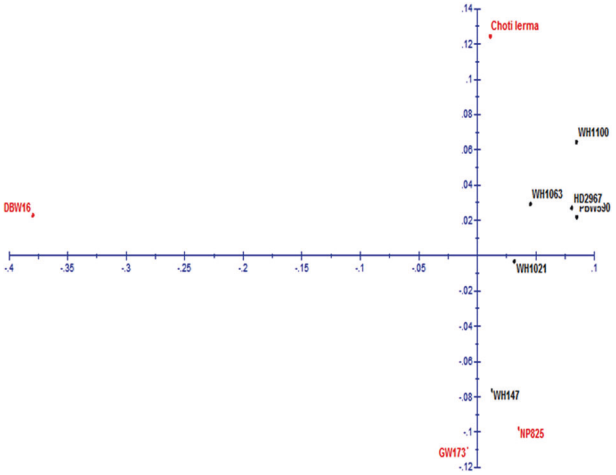

Fig. 7 2-D plot of principal component analysis (PCA) for 10 wheat genotypes in response to N. Red and blue color indicates deficient and efficient N wheat genotypes respectively.

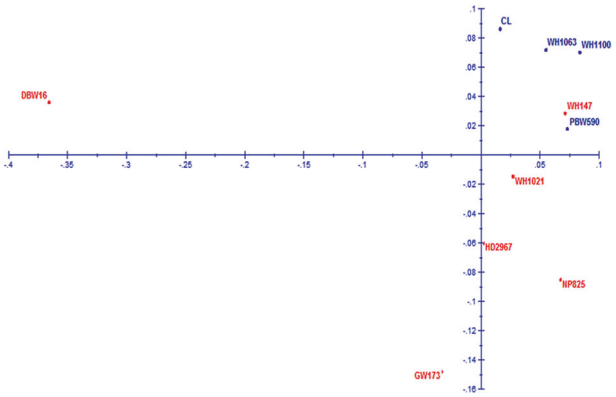

Fig. 8 2-D plot of principal component analysis (PCA) for 10 wheat genotypes in response to P. Red and blue color indicates deficient and efficient P wheat genotypes respectively.

candidate markers (miR167a and miR171a) showed high correlation between genotypic and trait data. miR171a was found to be highly associated with phenotypic data recorded under N conditions as miR171a showed presence of allele in N deficient and absence in N efficient genotypes. Likewise, miR167a showed

Table 2. Genetic diversity parameters of 2 sub populations of 10 wheat genotypes in response to Nitrogen.

Pop	N	Na	Ne	I	He	UHe	%P
POP1	5.000	2.438	2.036	0.746	0.465	0.517	93.75
POP2	5.000	1.875	1.648	0.523	0.358	0.397	84.38
Mean	5.000	2.156	1.842	0.634	0.411	0.457	89.06

Table 3. Genetic diversity parameters of 2 sub populations of 10 wheat genotypes in response to Phosphorous.

Pop	N	Na	Ne	I	He	UHe	%P
pop1	4.000	2.148	1.901	0.645	0.417	0.476	85.19
pop2	6.000	2.000	1.705	0.551	0.362	0.395	81.48
Total	5.000	2.074	1.803	0.598	0.389	0.436	83.33

Na number of different alleles, *Ne* number of effective alleles, *He* expected heterozygosity, *I* information index, *UHe* unbiased heterozygosity, *%P* polymorphic percentage.

Table 4. AMOVA calculated for the variance of 10 wheat genotypes in response to nitrogen.

Source	df	SS	MS	Est. Var.	%
Among Pops	1	17.600	17.600	1.029	12
Within Pops	18	131.600	7.311	7.311	88
Total	19	149.200		8.340	100

df Degree of freedom, *SS* Sum of squares, *MS* Mean sum of squares, *Est. var.* Estimated variance, *%* Percentage.

Table 5. AMOVA calculated for variance of 10 wheat genotypes in response to phosphorus.

Source	df	SS	MS	Est. Var.	%
Among Pops	1	13.133	13.133	0.768	12
Within Pops	18	103.667	5.759	5.759	88
Total	19	116.800		6.527	100

df Degree of freedom, *SS* Sum of squares, *MS* Mean sum of squares, *Est.var.* Estimated variance, *%* Percentage.

presence of allele in P efficient genotypes and absence of allele in P deficient genotypes.

DISCUSSION

Wheat crop does not perform well under N starvation conditions but responds well when there is an adequate level of N (Vinod and Heuer 2012). Out of the total amount of N taken up by wheat plant, 70% contained in seed, and the remaining 30% is contained in straw. In fact, crop plants use the N in two stages (i) N uptake that operates the growth and yield potential, (ii) N remobilization to the grain that determines the grain protein content. When soil is deficient in N, both the protein content and yield get reduced (Hawkesford 2012; Balyan et al. 2016).

Apart from N, P also plays a crucial role in wheat development. It has liability in root development, grain filling, and tillering stage (Hasan et al. 2016). Wheat with healthy root system and good vigor will be able to absorb the nutrients and water efficiently from the soil (Kumar et al. 2018b). N as nutrient helps the wheat plants to grow vigorously, however without adequate P levels, optimum N will not be able to produce a promising yield of crop.

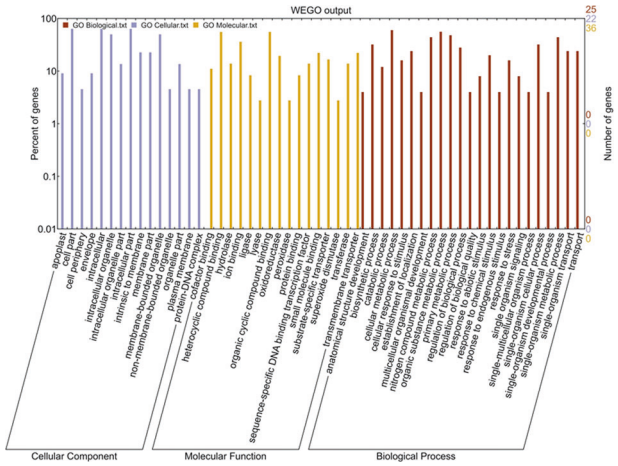

Fig. 9 The WEGO output for GO of the N and P responsive miRNAs targeted genes in wheat. The *x*-axis of plot showed three GO categories, whereas left and right side of *y*-axis represent the percentage of target genes and number of miRNA target genes respectively.

P is mobile in all plant parts and required at the early developmental stage of wheat (Mehta et al. 1963). P plays an essential role in plant growth stages such as the flowering stage, seed production, root development, and at maturity stage (Adnan et al. 2020). In P deficient soil, plants show deficiency symptoms such as stunted growth, reduction in number of tillers, purple stems, and roots (Marschner et al. 1996). Therefore, these nutrients are applied to soil in the form of fertilizers to achieve a good start, high yield, and protein quality. But grain crops are found to recover only one-third of applied fertilizers, whereas the rest are lost in the environment through surface run off and leaching (Hawkesford 2014). Therefore, adoptions of strategies for the improvement of NUE/PUE of wheat varieties are needed.

Among all the molecular markers, SSRs are mostly used for marker-assisted selection, QTL mapping, and genetic diversity analysis as they are distributed throughout the genome (Gupta et al. 1999). Most of SSRs were obtained from un-translated and protein-coding regions but the presence of SSRs in non-coding regions of miRNAs genes has limited data (Min et al. 2017). Many studies have been conducted on miRNA SSRs in response to various abiotic stresses (Sihag et al. 2021; Tyagi et al. 2021; Kumar et al. 2017a; Zhang et al. 2019). These miRNAs regulate the gene expression through cleavage or post-transcriptional modification by binding to complementary sequences in target gene (Kim 2005). These validated target genes may be transcription factor, signaling molecule that plays a vital role in development, physiological processes, and in abiotic stresses (Li et al. 2016; Hou et al. 2020).

PIC value is a good measure to calculate the polymorphic information of each marker. Markers having PIC value >0.5 are more informative as compared to markers with PIC value < 0.25 (Botstein et al. 1980). In the present study, the 35 polymorphic markers showed PIC values between 0.19–0.99 with an average PIC value of 0.72. Thus, our study showed higher polymorphism than the previous studies (Singh et al. 2018; Mondal and Ganie 2014; Tyagi et al. 2021).

Binary scoring data for N and P responsive polymorphic markers clustered the 10 wheat genotypes in three clusters (both for N and P) on the basis of their genetic background. Mondal and Ganie (2014) clustered the 24 rice genotypes in 4 clusters on the basis of salt sensitivity. Sihag et al. (2021) clustered the 20 wheat genotypes into three clusters by using the 19 polymorphic heat-responsive miRNAs SSRs. Furthermore, Tyagi et al. (2021)

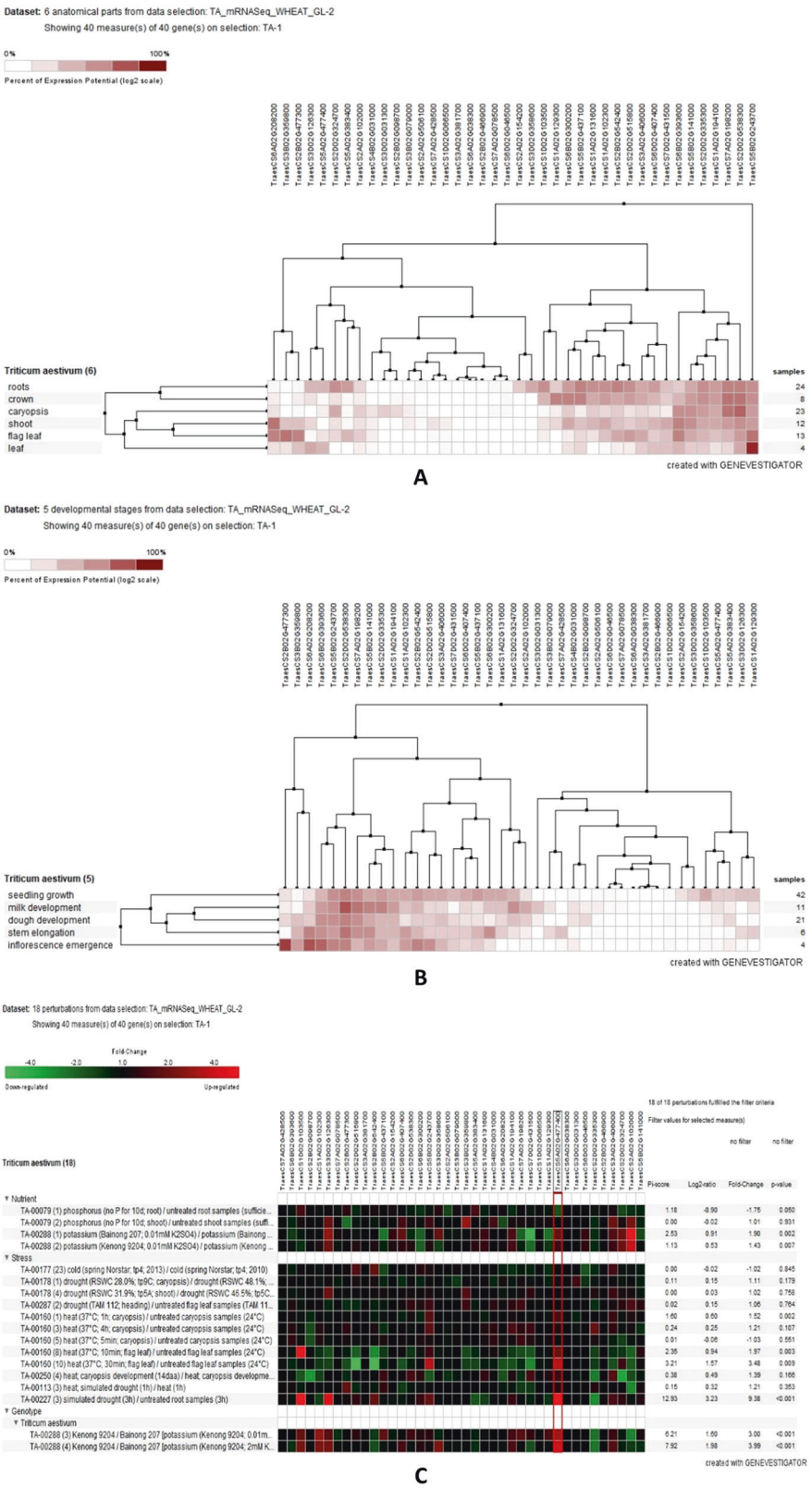

Fig. 10 Expression profiling of P responsive miRNAs targeted genes in wheat and their hierarchical clustering on the basis of their expression. A Anatomical parts, **B** development stages, and **C** under different P starvation conditions and abiotic stresses.

performed the diversity analysis with seven polymorphic SSRs on a panel of 37 wheat genotypes which grouped them into 4 clusters, out of which one cluster contained both the heat tolerant and sensitive genotypes.

In the present study, AMOVA results indicated that largest proportion of genetic variation was within-population (88%), whereas only 12% variation was among the populations, indicating that the major source of variation is the variability of

Table 6. Mean table of yield and its related traits data of 10 wheat genotypes under optimum and half dose of N.

Traits	Grain yield(gm)		Harvest Index		Biomass/plant (gm)		NUE	
Treatments→	Optimum	Half	Optimum	Half	Optimum	Half	Optimum	Half
All genotypes	16.86	11.88	37.03	30.92	19.24	14.33	38.96	45.35
N efficient genotypes	20.33	14.45	40.34	30.32	23.97	17.14	41.14	49.40
N deficient genotypes	13.39	9.30	33.72	31.53	14.51	11.53	36.76	41.30

Table 7. Mean table of yield and its related traits data of 10 wheat genotypes under optimum and half dose of P.

Traits	Grain yield (gm)		Harvest Index		Biomass/plant (gm)		PUE	
Treatments→	Optimum	Half	Optimum	Half	Optimum	Half	Optimum	Half
All genotypes	16.74	9.24	37.32	29.50	19.07	10.63	32.81	33.82
P efficient genotypes	19.87	10.61	41.13	32.60	22.90	11.17	35.14	36.00
P deficient genotypes	14.70	8.82	34.80	27.46	16.47	10.23	30.98	31.45

genotypes. Similar results of AMOVA were reported with 97% variation within the population by Tyagi et al. (2021). Shen et al. (2017) performed AMOVA on the data of 23 polymorphic SSRs that were screened over the 11 populations in *Xanthoceras sorbifolia* and revealed that 67% of total genetic variability accounted by variation between the population.

In silico expression profiling revealed the role of miRNA target genes in nutrient metabolism, growth, and development of wheat. As the data for N responsive miRNA target genes was not available at Genevestigator, therefore we searched for P responsive miRNA target genes in transcriptomics data at nutrient and various stress levels. Expression of these genes showed their role in five developmental stages and six anatomical parts of wheat. In our study, NAC transcription factor was found to be up-regulated under P starvation and environmental stresses, which confirmed the role of NAC in nutrient remobilization as reported by Borrill et al. (2017). In an earlier study, in silico expression profiling of *NLP* transcription factor was done in rice under N stress by using Genevestigator and NCBI-GEO. Expression of five *NLPs* was analyzed in 22 tissues and developmental stages, deciphered their role in nutrients, drought, hormonal, and salt stresses (Jagadhesan et al. 2020). Similarly, In silico expression of *TaRKD* and *TaNLP* genes were analyzed in wheat in fifteen anatomical parts and ten different developmental stages under various doses of nitrogen (Kumar et al. 2018b). Mehta et al. (2021) studied the tissue and stage-specific gene expression of 161 salt responsive genes in wheat by using Genevestigator database.

In an earlier study, it has been reported that miR171 and miR160 work in signaling pathway in root development in Arabidopsis. miR171 targets the SCL (SCARECROW-LIKE TF) and hence reduces the primary root elongation. Quantitative RT-PCR showed that miR171 up-regulated under N starvation condition and its expression was three-fold higher under N deficient condition as compared to N sufficient condition (Liang et al. 2012). In barrel medic, miR171 targets the *Nodulation Signaling Pathway 2 TF (NSP2)* in response to cytokinin (Ariel et al. 2012). A semi-quantitative and quantitative RT-PCR analysis disclosed that nine miRNAs including miR167a respond under P deficient conditions. miR167a up-regulated under P starvation and targets the auxin response factor 8 (Zhao et al. 2013). These recent findings verified our results that two miRNA families, miR171a and miR167a, play important role in the nutrient starvation condition in distinct plant species. miR171a can discriminate between N efficient and deficient wheat genotypes, whereas miR167a can distinguish the P efficient and deficient wheat genotypes. Accordingly, these miRNAs-based SSRs proved to be useful in breeding program to discriminate the wheat genotypes in N and P efficient and deficient conditions. Analysis of phenotypic data of all the 10 wheat genotypes also indicated that the marker miR171a and miR167a showed the linkage with N/PUE as well as with trait related data.

Excessive use of N and P fertilizers causes the environmental degradation and multi-nutrient deficiencies in soil which ultimately affects the wheat yield. In order to reduce the use of these fertilizers, it becomes necessary to develop the wheat varieties with high N and P use efficiency without compromising its yield. In this study, we identified 35 miRNA and genic SSRs in response to N and P. Out of these, only two miRNA SSRs- miR171a and miR167a were found to distinguish the N and P efficient and deficient wheat genotypes, respectively. These molecular markers will be highly informative and helpful in breeding programs to develop the N and P efficient varieties, which will further help in reducing the consumption of chemical fertilizers and their harmful effects on the environment without affecting the yield of wheat.

DATA AVAILABILITY

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author/s.

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AUTHOR CONTRIBUTIONS

UK designed the experiments and obtained funding for this research. VS and PS conducted the experiments. VS, AK, YS, SM, PK, and PB performed data analysis. VS and UK drafted the manuscript. RM and OPD revised the manuscript. All authors agreed to the final manuscript.

COMPETING INTERESTS

The authors stated that this study was conducted in the absence of a commercial or financial relationship that could be considered a potential conflict of interest.

ADDITIONAL INFORMATION

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