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Article

Combined Genome-Wide Association Studies (GWAS) and Linkage Mapping Identifies Genomic Regions Associated with Seedling Root System Architecture (RSA) under Different Nitrogen Conditions in Wheat (*Triticum aestivum* L.)

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Abstract: The nutrient absorption and utilization efficiency in the roots of seedlings are beneficial for increasing crop yield. Creating marker assisted selection for wheat root traits can assist wheat breeders in choosing robust roots to maximize nutrient uptake. Exploring and identifying the effect of different nitrogen applications on RSA is of great significance for breeding nitrogen efficient wheat varieties. In this study, A total of 243 wheat varieties native to the Yellow and Huai Valley regions of China were utilized for genome-wide association studies (GWAS). Furthermore, a recombinant inbred line (RIL) population of 123 lines derived from the cross between Avocet and Chilero was utilized for linkage examination and confirmation of the hereditary loci distinguished by GWAS. Five RSA traits include the relative number of root tips (RNRT); relative total root length (RTRL); relative total root surface area (RTRS); relative total root volume (RTRV) and relative average root diameter (RARD) where evaluated under two N conditions—normal N (NN) supply and low N (LN)—to characterize the NUE of each accession. GWAS and linkage analysis were performed by integrating data from the wheat 660k single nucleotide polymorphism (SNP) chip and diversity arrays technology (DArT) to identify genetic loci associated with RSA. The results showed that based on the ratio of RSA related traits under two N supply conditions, a total of 598 SNP markers, which are significantly associated with RSA related traits, were detected at 160 genetic loci by GWAS. A total of 10 QTL loci related to RSA were discovered and identified by linkage mapping. In which, three significant RSA related Quantitative Trait Locus (QTL) were identified, namely qRtr1-3D, qRnrt-3D, and qRtr1-7D. The study has the potential to enhance the effectiveness of selecting root traits in wheat breeding programs, offering valuable insights into the genetic underpinnings of nitrogen use efficiency (NUE) in wheat. This could lead to the creation of NUE wheat varieties by implementing focused breeding strategies

Keywords: *Triticum aestivum* L; root system architecture; N use efficiency; GWAS; linkage mapping; 660K SNP chip; DArT

1. Introduction

Wheat stands as the most extensively cultivated grain globally, with 20% of the world's human dietary intake of calories and protein. By 2050, the world's population is projected to exceed 9 billion, leading to a 60% increase in wheat demand as compared to 2020 [1]. N is a key component of various basic functions of plants, such as chlorophyll synthesis, protein formation, nucleic acid production, enzyme activity, alkaloid synthesis, hormone regulation, and vitamin synthesis, all of which play important roles. N is usually the main yield-limiting factor in crop production. Properly using nitrogen fertilizer can greatly enhance crop yield, while overapplying it not only raises production costs but also leads to significant environmental pollution issues. Enhancing nitrogen use NUE of

wheat emerges as a sustainable approach to achieve environmentally friendly and safety of wheat production. This highlighted the significance of breeding wheat varieties with high NUE in China. Various research studies have demonstrated that the efficiency of crop NUE largely depends on RSA, and the presence and spread of external nitrogen sources can, in return, modify the RSA of crops [2]. Therefore, the wheat RSA response to external N levels may represent a pivotal avenue to enhance NUE. Historically, breeders have primarily assessed NUE based on above-ground phenotypic traits, over-looking the critical role of underground roots. This limited perspective has hindered advancements in high-yield wheat breeding to some extent [3–6].

Many studies have shown that the RSA is crucial in the growth and development of wheat [3,7,8]. RSA encompasses the structural and spatial characteristics of the root system, encompassing traits such as total root length (TRL), total root surface area (TRS), total root volume (TRV), number of root tips (NRT), and average root diameter (ARD) [3,5]. RSA is intricately linked to physiological aspects like N absorption and transport [4,6,9], and exhibit a significant positive correlation with plant N utilization capacity [6,10]. Thus, RSA has been used as an important index for associating N efficient varieties in rice [11], wheat [12,13], and maize [14,15].

RSA is a common complex quantitative trait influenced by multiple genes. Combined GWAS and linkage map-ping are powerful tools for detecting genes and linkage analysis under complex traits [16]. Because of the restricted genetic diversity in the parental populations, numerous QTLs are unable to be identified [17–19]. Compared with linkage analysis, GWAS are more efficient and provide higher resolution in identifying gene loci. Moreover, combining linkage analysis with GWAS can improve the credibility of the results. This approach has been used to study agronomic characteristics, abiotic stress factors, and genes related to disease resistance in maize [20], cucumber [21], and Brassica napus [22]. In the two latest studies on drought resistance in winter wheat, GWAS and linkage analysis were simultaneously used to identify genomic regions related to drought resistance [23,24]. Herein, our study conducted a comprehensive analysis of the natural population for GWAS, and the RIL population was utilized for link-age examination and confirmation of the hereditary loci distinguished by GWAS under the conditions of normal N (NN) and low N (LN) supply, and the important gene regions related to N response in wheat were identified through GWAS and linkage analysis. Study on RSA related traits of different genotypes at seedling stage under two nitrogen hydroponic environments of NN and LN. Different characteristics of RSA were analyzed to pinpoint genes associated with NUE, offering insight for developing high NUE wheat cultivars.

2. Materials and Methods

2.1. Plant Materials

This study utilized two panels made of wheat. Panel I: A total of 243 wheat varieties native to the Yellow and Huai Valley regions of China were utilized for GWAS (Table S1) [25]. Panel 2: The RIL population of 123 lines derived from the cross between Avocet and Chilero, both of them are the wheat backbone parent from the International Maize and Wheat Improvement Center (CIMMYT). Using the single seed descent method [26]. It was previously utilized to describe resistance to leaf rust and stripe rust [27]. Our study found that the RSA of Avocet and Chilero were quite different in NN and LN environments. The seeds of the parents and the RIL population were utilized for linkage examination and confirmation of the hereditary loci distinguished by GWAS.

2.2. Experimental Design and Trail Management

The nutrient solution culture method was used in a seedling stage test. The nutrient solution was referred to Hoagland et al. [28] nutrient solution and appropriately modified according to the nutritional characteristics of wheat (Table 1). Two treatments of normal N (NN, nutrient solution formula is shown in Table 1) and low N (LN) were set in the laboratory. The nutrient solution difference was N supply with N in LN treatment are 0.4 mmol/L $\text{Ca}(\text{NO}_3)_2$ and 0 mmol/L $(\text{NH}_4)_2\text{SO}_4$. The uniform wheat grains were soaked in a 10% H_2O_2 solution for 10 minutes, then rinsed

three times with deionized water before being placed on wet filter paper for culturing. Once the seedlings reached the one-leaf stage and exhibited similar growth, they were fixed in seedling trays and grown in NN and LN nutrient solutions. The nutrient solutions were aerated continuously with an oxygen pump, and changed every 3 days.

Table 1. Components of nutrient solution for wheat seedling culture.

Macronutrients	Concentration(mmol/L)	Trace Elements	Concentration(mmol/L)
KCl	1.8	H ₃ BO ₃	1×10 ⁻³
CaCl ₂	1.5	ZnSO ₄	1×10 ⁻³
Ca(NO ₃) ₂	1	MnSO ₄	1×10 ⁻³
(NH ₄) ₂ SO ₄	1	CuSO ₄	0.5×10 ⁻³
MgSO ₄	0.5	Fe-EDTA	0.1
KH ₂ PO ₄	0.2	(NH ₄) ₆ Mo ₇ O ₂₄	1.0×10 ⁻⁴

2.3. Trait Measurements

Following a 21 days of cultivation, the roots were washed with deionized water before being trimmed at the segmented points using scissors. Which were placed in a transparent tray for testing. Five individual plants were randomly selected from each sample, and the lateral roots of each plant were separated one by one, placed in a transparent root tray containing deionized water in a non overlapping state, and the root system was scanned by the WinRHIZOLA6400XL perspective scanning system to obtain the root system pictures of each individual plant. Then the WinRHIZOPro software was used to analyze the phenotypic data of each sample, such as TRL, TRS, TRV, NRT, and ARD, and the phenotypic data value of the material was determined by calculating the average of three replicates Subsequently, the relative values of each trait under two different N conditions were calculated, namely, RNRT, RTRL, RTRS, RTRV and RARD. Origin 2021 was used to statistically analyze the data, and the correlation coefficient was calculated.

2.4. Principal Component Analysis (PCA) and Kinship Analysis

With the aid of Tassel v5.0 (<https://www.maizegenetics.net/tassel>), PCA and kinship analysis were carried out.

2.5. GWAS

The 660k gene chip data of the natural population were analyzed by Beijing CapitaBio Technology Co. Ltd (<http://capital.en.drugdu.com/>). in the study of Fusarium nipponense crown rot resistance of wheat previously used. After data quality check, 395782 SNP markers in total were kept [25]. The mixed linear model (MLM) in Tassel V5.0 software was used for GWAS. The model principle is as follows ([25,29,30].

$$Y=X\alpha+Q\beta+K\mu+\varepsilon,$$

Phenomenal attributes are denoted by Y, genotype by X, and principal components matrix (Q) by the first three principal components; The kinship matrix is K; corresponding effects are α . β and μ ; residual effects are represented by the matrix ε . The matrices X and Q were regarded as fixed effects, while the matrices K and ε were regarded as random effects.

When the SNP marker -log10 (P-value) > 5, it is considered that there is a significant association between the marker and the trait. Based on the attenuation distance of whole genome linkage disequilibrium (LD), the 10MB interval before and after significant SNPs were identified as QTL sites [25]. Using the CMplot program in the R package, manhattan and quantile quantile (Q-Q) plots were made.

The MLM model in Tassel V5.0 software was applied to analyze the correlation of root architecture of the natural population with PCA and kinship as covariates, combined with the

screened genotype data. In the results of GWAS analysis, when $\text{Log}_{10}(P\text{-value}) > 5$, there was a considerable correlation between the marker and the characteristic. Using the R package CMplot program, Manhattan plots and Q-Q plots were visualized.

2.5. Linkage Analysis

Zhao's genetic linkage map data [31] indicates that. After screening and de-redundancy, a high-density genetic linkage map was created from the whole genomes of the RIL population and its two parents using a wheat DArT array. The map covers 21 wheat chromosomes and is 8202.10 cM long. It features 3627 DArT markers spaced 2.26 cM apart on average. The QTL of the RSA trait was mapped using the compound interval mapping approach with the LOD threshold set to 2.5 using the BIP function of the QTL IciMapping V4.2 program. MapChart V2.32 software was used to map the QTL linkage group. QTLs that shared flanking markers or had a spacing of less than 1 cM were considered to be at the same locus [32]. The QTL names were assigned using McCouch's methodology [33].

3. Results

3.1. Phenotypic Evaluation

The results showed that in the two populations, different RSA indexes of wheat showed certain variability, and the variation range of the variation coefficient was quite different. Under the NN condition, the variation coefficient of each character of the natural population and the RIL population ranged from 11.39% (ARD) -39.72% (TRL) and 14.29% (ARD) -48.98% (TRS), while under the LN condition, the variation coefficient of each character of the natural population and the RIL population ranged from 26.36% (ARD) -54.19% (NRT) and 16.39% (ARD) -55.09% (TRS) Ln conditions. Comparing the relative values, it was found that the relative values of all RSA traits were greater than 1 except RARD (0.98) in the natural population. The variation coefficients of each trait in the natural population and the RIL population ranged from 29.24% (RARD) -52.26% (NRT) and 14.78% (RARD) -46.62% (RNRT).

The RSA traits of the natural population and the RIL population showed continuous distribution under two N supply conditions, and the genetic basis was rich, which was appropriate for linkage analysis and GWAS analysis and linkage analysis (Figure. 1). The correlation analysis of RSA traits showed that LN supply could increase TRL, TRS and NRT, and the increase was more obvious in the natural population (Table S1, Table 2). Moreover, Figure 2 shows that the proportion of TRL, TRS, TRV, and NRT were significantly positively correlated in two populations and two N supply conditions ($P < 0.05$), with the correlation coefficient r ranging from 0.19 to 0.95. However, in the natural populations, the ARD was significantly negatively correlated with TRL and NRT in two N supply conditions ($P < 0.05$), and the correlation coefficient r ranging from -0.46 to -0.18, while in the RIL population, it was only negatively correlated in NN treatment ($P < 0.05$).

The T-test results of the RIL population parents showed that under the NN environment, the TRL, TRS, TRV, and NRT of Avocet were all greater than Chilero ($P < 0.05$), however, there was an opposite tendency under the LN condition ($P < 0.05$). This reflects the significant differences in the RSA responses of parents Avocet and Chilero under low nitrogen stress. (Figure. 3).

Table 2. Summary of RSA traits in different populations under different nitrogen levels.

Treatm ent	Trait	natural population					RIL population				
		Mi n.	Ma x.	Avera ge	Stand ard deviati on	CV (%)	Mi n.	Max .	Avera ge	Stand ard deviati on	CV (%)
NN nutrien t solutio n	TRL (cm)	60. 07	372 .1	143.72	57.09	39. 72	58. 19	279. 43	132.28	53.46	40. 41
	TRS (cm2)	14. 13	135 .1	51.73	19.59	37. 87	19. 29	209. 41	45.27	22.16	48. 95
	ARD (mm)	0.6 7	1.6 2	1.15	0.13	11. 39	0.8 4	2.3	1.08	0.15	14. 29
	TRV (cm3)	0.2 5	3.9	1.5	0.58	38. 95	0.4 5	2.52	1.18	1.08	37. 14
	NRT	38	293	91	35.6	39. 2	23	214	81	33.16	41. 15
LN nutrien t solutio n	TRL (cm)	80. 12	474 .4	237.38	89.54	37. 72	66. 91	290. 3	146.09	55.6	44. 09
	TRS (cm2)	20. 3	159 .3	77.71	27.42	35. 29	18 67	232. 67	44.35	24.43	55. 09
	ARD (mm)	0.2 3	2.3 1	1.11	0.29	26. 36	0.8 4	2.55	1.13	0.19	16. 39
	TRV(c m3)	0.0 9	4.5 4	1.91	0.85	44. 62	0.3 8	2.48	1.17	1.32	40
	NRT	43	457	134	72	54. 19	29	171	82	33	39. 88
Relativ e ratio	RTRL	0.9 3	5.6 8	1.8	0.83	45. 88	0.3 1	2.45	1.05	0.46	44. 34
	RTRS	0.7 9	5.6 8	1.62	0.69	42. 8	0.3 4	2.42	1.06	0.46	42. 95
	RARD	0.2	2.2 2	0.98	0.29	29. 24	0.6 9	1.64	1.06	0.16	14. 78
	RTRV	0.0 6	5.6 1	1.4	0.78	55. 72	0.3 6	2.66	1.1	0.5	45. 57
	RNRT	0.7 3	5.5 8	1.56	0.82	52. 26	0.2 7	3.02	1.13	0.53	46. 62

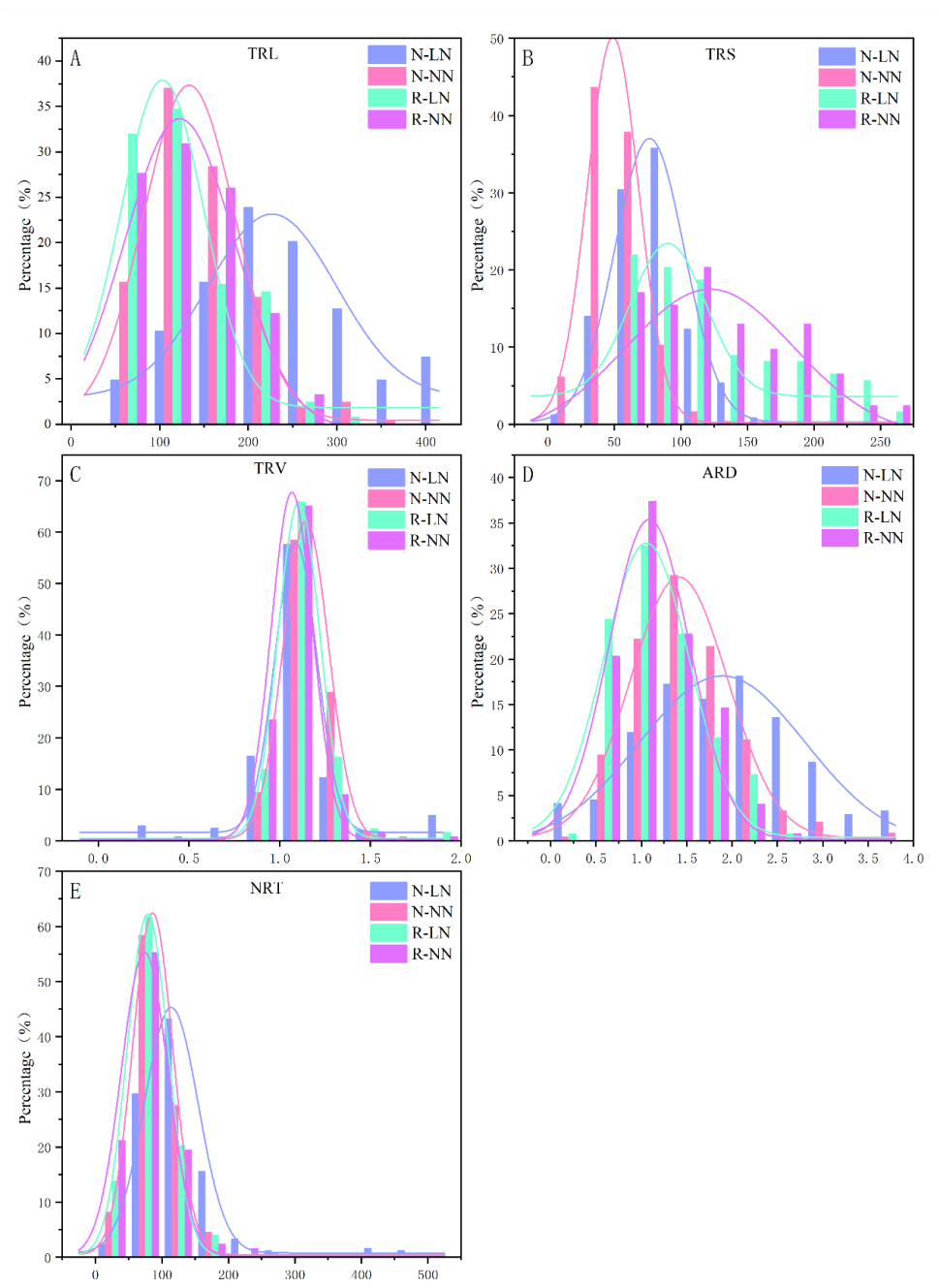


Figure 1. Phenotype distribution map of the natural population and the RIL population. N-LN: The natural populations under low N treatment; N-NN: The natural population under normal N treatment; R-LN: The RIL population under low N treatment; R-NN: The RIL population under normal N treatment.

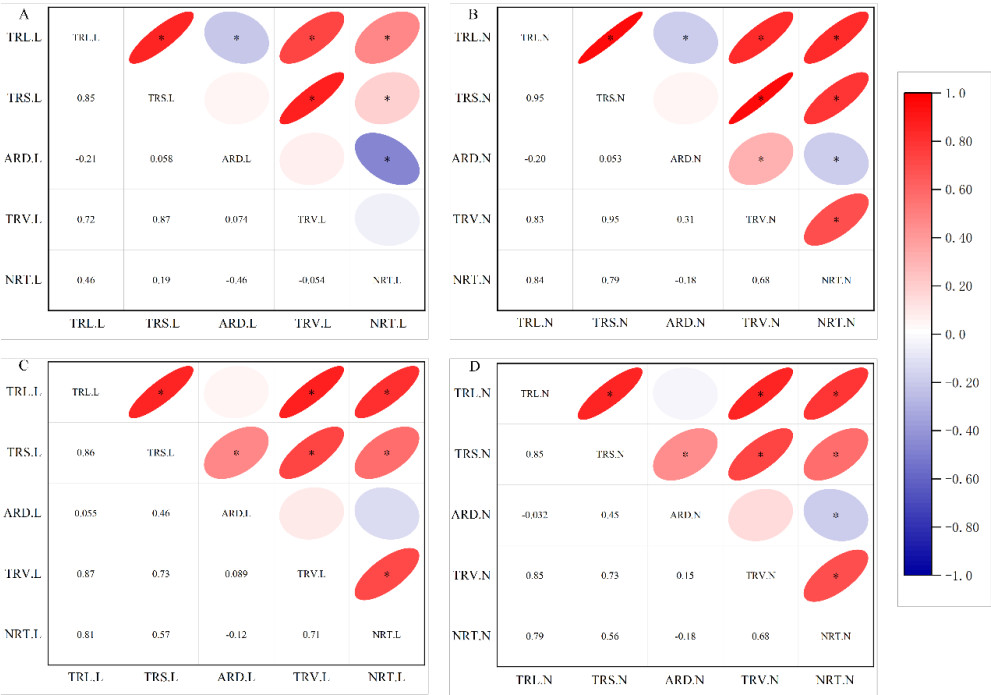


Figure 2. Correlation map between variables of natural population under LN treatment (A), natural population under NN treatment (B), the RIL population under LN treatment (C), and The RIL population under NN treatment (D). TRL.L: Total root length under LN treatment; TRL.N: Total root length under NN treatment; TRS.L: Total root surface area under LN treatment; TRS.N: Total root surface area under NN treatment; TRV.L: Total root volume under LN treatment; TRV.N: Total root volume under NN treatment; ARD.L: Average root diameter under LN treatment; ARD.N: Average root diameter under NN treatment; NRT.L: Number of root tips under LN treatment; NRT.N: Number of root tips under NN treatment.

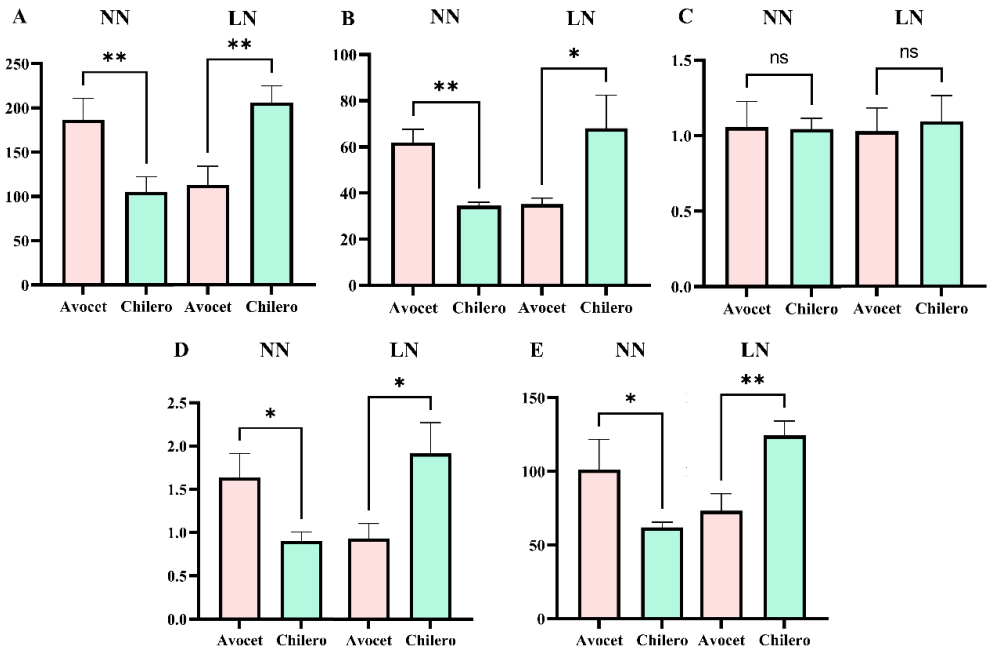


Figure 3. Effects of different treatments on RSA of Avocet and Chilero. Lowercase letters indicate significant difference among Avocet and Chilero ($P < 0.05$). * and ** indicate significantly difference at $P < 0.05$ and $P < 0.01$, respectively.

3.2. GWAS Analysis

The quantile-quantile (Q-Q) plots indicated that the false positives were well controlled in GWAS models (Figure 4). There were found to be 598 SNP markers that were substantially linked to features relevant to nitrogen utilization efficiency, distributed across 160 genetic loci, based on the ratios of RSA-related traits under two distinct nitrogen levels. (Figure.4 and Table S2). Ten loci were linked to RTRL, accounting for 8.0%–15.6% of the phenotypic variation; ninety loci were linked to RTRS, accounting for 9.1%–26.9% of the variation; sixty-seven loci were linked to RTRV, accounting for 9.1%–19.8% of the variation; sixty-three loci were linked to RARD, accounting for 8.2%–24.7% of the variation; and seventy-nine loci were linked to RNRT, accounting for 8.9%–15.2% of the phenotypic variation. There was a high correlation between the ratio of RSA traits under two N supply conditions. Correspondingly, in the association results, we found that multiple relative traits have the same genetic loci. Among these one locus is associated with RTRS and RTRV, four loci are associated with RTRS and RARD, four loci are associated with RTRS and RNRT, and three loci are associated with RARD and RNRT, one locus is associated with RTRS, RTRV, and RARD, 31 loci are associated with RTRS, RTRV, and RNRT, one locus is associated with RTRS, RARD, and RNRT, six loci are associated with RTRL, RTRS, RTRV, and RNRT, 12 loci are associated with RTRS, RTRV, RARD, and RNRT, and three loci are associated with RTRL, RTRS, RTRV, RARD, and RNRT.

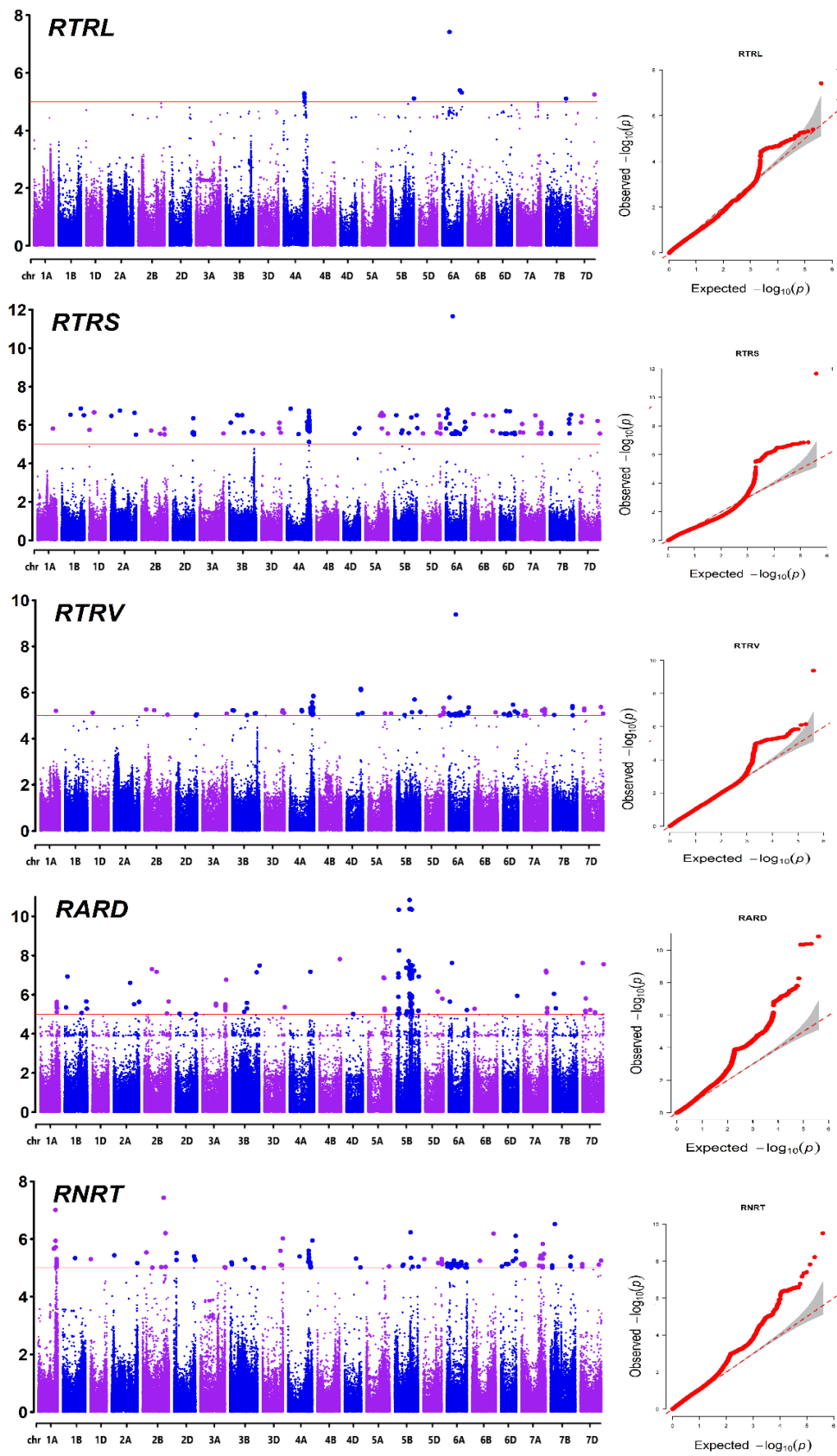


Figure 4. GWAS of relative values of RSA of natural population under different N conditions using Manhattan plot and QQ plot.

3.3. Linkage Analysis

Three QTLs in all were found to be linked to RTRL, and with LOD values ranging from 3.15 to 5.16, the unit point might account for 7.58%–15.04% of the phenotypic variation. The LOD values were 3.71-4.16 for the two QTLs that were determined to be linked to RTRS. These QTLs could account for 10.22%–15.92% of the variation in phenotype for each locus. Two QTLs in all were found to be linked to RARD, and with LOD values ranging from 2.52 to 3.95, the unit point might account for 8.16%–13.72% of the phenotypic variation. One QTL in all were found to be linked to RNRT, and with LOD values was 2.89, the unit point might account for 10.3% of the phenotypic variation (Figure 5 and Table 3).

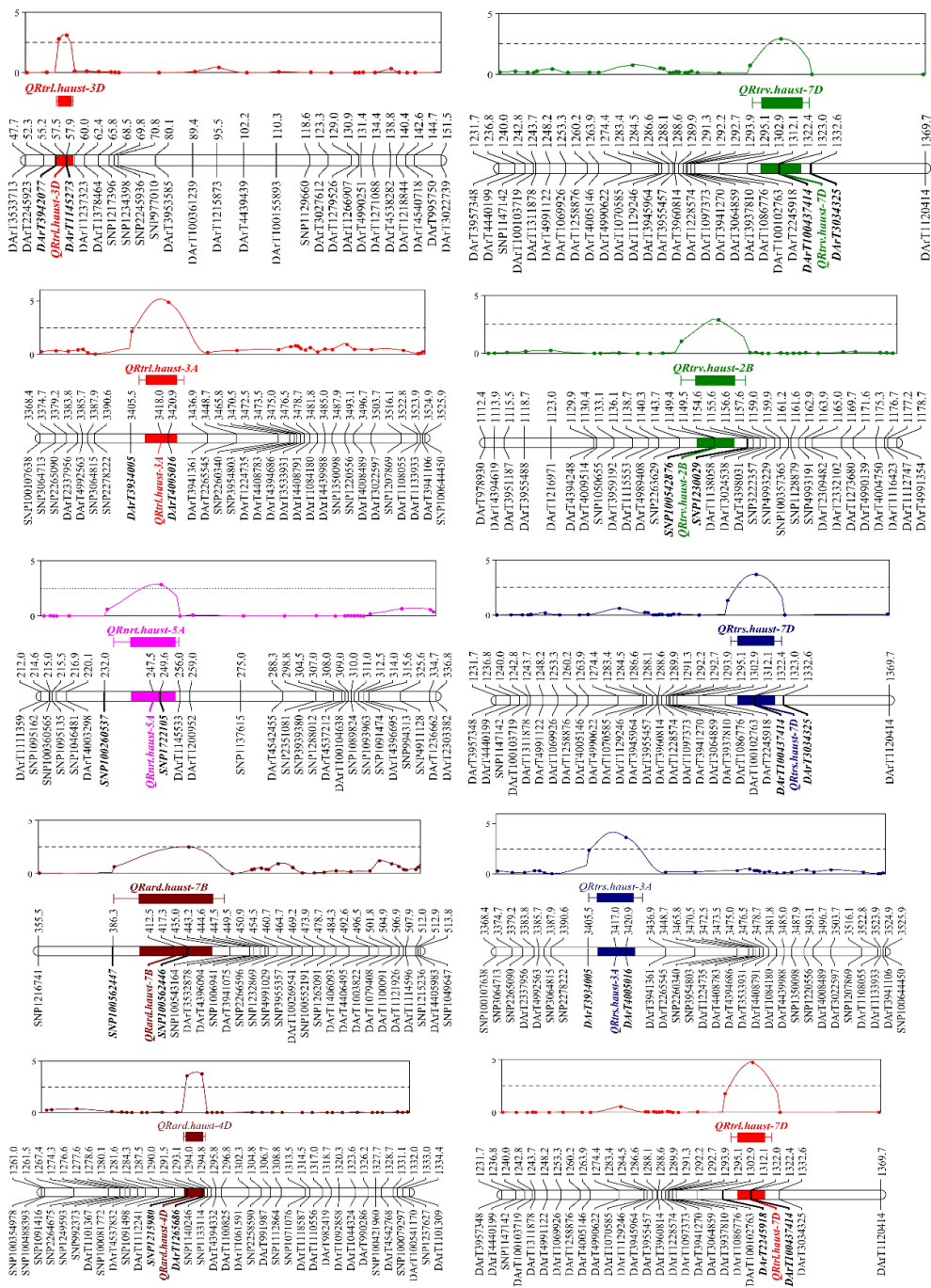


Figure 5. Distribution of QTLs related to RSA on chromosomes. .

Table 3. Linkage analysis results.

Trait Name	QTL	Position (MB)	Left Marker	Right Marker	LOD	PVE (%)	Add
RTRL	QRtrl.haust-3A	3418	DarT3934005	DarT4005016	5.16	15.05	-0.2315
RTRL	QRtrl.haust-3D	57	DarT2260219	DarT1145273	3.15	7.58	-0.142
RTRL	QRtrl.haust-7D	1322	DarT2245918	DarT100437414	4.76	11.23	0.1876
RTRS	QRtrs.haust-3A	3416	DarT3934005	DarT4005016	4.16	15.92	-0.2275
RTRS	QRtrs.haust-7D	1322	DarT2245918	DarT100437414	3.71	10.22	0.1725
RARD	QRard.haust-4B	1292	SNP1215980	SNP100022040	3.95	13.72	-0.0572
RARD	QRard.haust-6B	417	SNP100562446	SNP100562447	2.52	8.16	-0.0441
RTRV	QRtrv.haust-2B	1149	SNP2263629	SNP100542876	2.96	9.71	0.156
RTRV	QRtrv.haust-7D	1323	DarT100437414	DarT3034325	2.93	9.93	0.1743
RNRT	QRnrt.haust-5A	249	SNP100260537	SNP1722105	2.89	10.27	-0.176

3.4. Colocalized Gene Regions

Using GWAS and linkage mapping, three important RSA-related Quantitative Trait Locus (QTL) were found. (Table 4). The significant marker AX-95160997 obtained through GWAS analysis of RTRL loci is located within the significant QTL QRtrl.haust-3D obtained through linkage analysis, with a physical interval of 39.61–43.74 MB. The significant marker AX-109592379 associated with RNRT is located within the significance QTL QRnrt.haust-5A, with a physical interval of 649.97-661.55 MB. The significant markers AX-110924288 associated with RTRL and RTRS are located in QRtrl.aust-7D and QRtrs.haust-7D, with a physical interval of 592.44-605.36 Mb.

Table 4. Colocalized gene regions.

Trait Name	Chr	Loci	Physical interval (Mb)	PVE (%)
RTRL	3D	AX-95160997/QRtrl.haust-3D	39.61-43.74	7.58-9.14
RNRT	5A	AX-109592379/QRnrt.haust-5A	649.97-661.55	10.27-10.81
RTRL,RTRS	7D	AX-110924288/QRtrl.haust-7D/QRtrs.haust-7D	592.44-605.36	9.95-11.22

4. Discussion

4.1. Effect of RSA on NUE

Wheat yield is significantly impacted by RSA [2]. Breeders partially completed the selection of wheat root architecture by indirect means during the artificial breeding process to aggregate high-yield genes and choose high-yield materials [34]. In this trial, we found that the RSA were different among materials of the two wheat populations, and there was a high correlation between the ratio of RSA traits under two N supply conditions. Therefore, it is very necessary to indirectly explore NUE related loci by carrying out the identification of RSA under different N conditions to promote the selection and breeding of wheat with high NUE.

The developed root system is the basis of efficient N absorption and utilization. The more developed the root, the thinner the average diameter of the root [35]. The more fine roots, the more root tips, the longer the TRL, and the larger the TRS are, the more conducive it is for crops to effectively capture N in the soil [36,37]. Crops with different N efficiency not only have different performance in dry matter weight and N accumulation, but also have different root morphological and quantitative properties [2,38]. Numerous research has demonstrated that wheat that is N efficient has higher TRS [2,39]. N efficient rice had substantially higher TRL, TRS, and TRV than N inefficient

rice [40,41]. Cassman et al [42] discovered that the N efficient rice's TRV offered more benefits. These findings suggested that the roots of the N efficient genotype were more developed.

This study demonstrated that reduced N supply enhanced the TRL, TRS, TRV, and NRT of seedlings compared to normal N supply, which is consistent with the prior research results [2,38]. Moreover, the change amplitude of natural populations is more obvious than the RIL populations, which may be related to the richer genetic diversity of natural populations. This proves that the elongation of most materials' roots can be promoted under low nitrogen stress. Besides ARD, all coefficient of variations (CV) are greater than 20%, indicating that this is closely related to the genotype of the material. Moreover, through correlation analysis, this study found that ARD was negatively correlated with TRL, TRS, TRV and, NRT, suggesting that the average diameter of roots is smaller in better developed roots. Crops have an easier time efficiently absorbing nitrogen from the soil when there are more fine roots, more root tips, longer total root length, and greater root absorption area. Which is in line with the findings of earlier studies [35,36]. Moreover, through correlation analysis, this study found that ARD was negatively correlated with TRL, TRS, TRV, and NRT, which indicated that the more developed roots are, the thinner the average diameter of the roots is. The more fine roots, the more root tips, the longer the total root length and the larger the root absorption area are, the more conducive it is for crops to effectively capture nitrogen in the soil. Which is consistent with previous research results.

To mine QTL loci relevant to nitrogen efficiency, the response of wheat root RSA to low nitrogen stress was investigated in this work using the relative value approach of various nitrogen circumstances. This approach has been widely utilized to mine wheat NUE genes [2,43]. It can more correctly mine RSA genes affected by low nitrogen and better depict the reaction of plant seedling roots to low nitrogen environment. The CV of the relative proportion of RSA of the natural population and the RIL population is more than 40% except for RARD, which proves that under low nitrogen stress, there are great differences in RSA of wheat genotypes, and different genotypes have different responses to low nitrogen.

4.2. Linkage Analysis and GWAS together Offer a Novel Method for Identifying the Genes Causing RSA

Linkage analysis, sometimes referred to as QTL mapping, is a common technique for examining how quantitative traits are inherited. QTL loci for traits related to NUE were mapped under field conditions, however, relatively limited genetic variation in the parental population resulted in a large physical distance [44,45]. To reduce QTL intervals by raising marker density inside candidate segments, fine mapping is typically necessary [46,47]. Compared with traditional linkage analysis, GWAS takes natural populations as materials, with a wide range of sources (wild species, local varieties, modern varieties, and high generation strains), high gene polymorphisms, and no need to build a parental population.

Its benefits include low cost, excellent resolution, and high efficiency. The process of genetically analyzing quantitative traits has been greatly aided in recent years by the invention and application of wheat gene chips [48]. Numerous QTLs and QTL clusters have also been found in various wheat N treatment trials [2,38,49–54]. In this study, the wheat 660k SNP chip and DArT technology were used to analyze the inheritance of RSA during the wheat seedling stage employing GWAS and QTL mapping under different N levels. We found that the total number of loci significantly associated with RSA is distributed on 21 chromosomes. Some of these loci overlapped or coincided with multiple RSA related loci discovered by Jin et al. [2], Xiong et al. [38], Fan et al. [54], Meng-jiao Yang et al. [52], and Ren et al. [53] (Table S3). In which the mapped area contains numerous known NUE related genes such as NRT2s genes on chromosome 6A, which can be associated with RTRL, RTRS, RNRT, and other traits. In addition, NPF6.2, FD-GOGAT, and the GWAS and QTL mapped region contain the GS2 genes [55–57].

Combined GWAS and linkage mapping, a significant locus *AX-95160997/QRtr1.haust-3D* (Table 4) was detected, which may be an important NUE locus. According to its colocalized physical location, it is between 39.61-43.74MB named as *qRtr1-3D*. In this interval, the Chinese Spring 2.0 database was used to screen high-confidence genes. There are a total of 61 annotated genes. Through

screening, we found a possible candidate gene *TraesCS3D02G090300*, which is located at 0.05Mb flanking the significance marker *DarT1145273* (*QRtrl.haust-3D*). By querying the gene annotation, we found that it encodes a MADS box transcription factor. The MADS box gene family plays an irreplaceable role in the process of plant growth and development and signaling. Studies have shown that the MADS box transcription factor is involved in regulating lateral root growth in Arabidopsis [58,59]. In Arabidopsis, the MADS box transcription factor is mainly expressed in roots and regulates lateral root development by regulating auxin synthesis in roots [60]. Recent studies have shown that, the MADS box transcription factor family is involved in regulating NUE in wheat and plays an important role in nitrate supply in roots, which provides new clues for further exploring the function of this gene [61,62].

This study also found two colocalized intervals *AX-109592379/QRnrt.haust-5A* and *AX-110924288/ QRtrl.haust-7D/QRtrs.haust-7D* (Table 4). According to the physical location of the colocalization of these two sites, it is between 649.97-661.55 MB and 592.44-605.36 MB, which is called *qRnrt-5A* and *qRtrl-7D*. Compared with previous studies, no overlapping interval was found, which may be two new loci. Candidate genes were screened in the *qRtrl-7D* interval, and some possible candidate genes were listed, such as the *TraesCS7D02G538000* gene, which encodes an E3 ubiquitin protein ligase. The multitype protein family E3 ubiquitin protein ligase is crucial for root and bud growth and development, as well as plant N absorption. [63,64]. Candidate genes were screened in the *q Rnrt-5A* interval, and some possible candidate genes were listed, such as the *TraesCS5A02G519300* gene, which encodes a protein with the NAC binding domain (Table 5). Studies have shown that overexpression of the NAC transcription factor significantly increases nitrate influx rate, N uptake, and other traits [65], but it cannot be identified as a candidate gene due to the large candidate interval.

NUE was significant differences among species or subspecies and different plant organs. With the release of wheat genome sequence, gene function verification and exploration have become an important topic in the post genome era of wheat. However, the widespread phenomenon of one cause and multiple effects in organisms also makes the determination of gene function difficult, which still requires solid gene function verification. At present, bioinformatic analysis based on reliable association markers is an effective method to explore candidate genes related to complex agronomic traits. To provide more candidate gene loci for N efficient molecular breeding, targeted improvement of specific crop subspecies or creation of excellent new varieties, and providing references for improving NUE, we should deeply explore the key regulatory genes and excellent allelic variation that control NUE different N application levels.

Table 5. Candidate gene prediction.

QTL	Gene	Position(Mb)	Gene annotation or coding protein
<i>qRtrl-3D</i>	<i>TraesCS3D02G083600</i>	40.0397-40.4099	F-box protein PP2-B11
	<i>TraesCS3D02G087200</i>	42.06815-42.07455	Rab GTPase-activating protein
	<i>TraesCS3D02G088200</i>	42.92659-42.93343	E3 ubiquitin-protein ligase ATL41
	<i>TraesCS3D02G088300</i>	42.92979-42.93343	Probable LRR receptor-like serine/threonine-protein kinase At3g47570
	<i>TraesCS3D02G088900</i>	42.97653-42.97697	Histone H2B.1
	<i>TraesCS3D02G090300</i>	43.79470-43.79640	MADS-box transcription factor 29

<i>qRnrt-5A</i>	<i>TraesCS5A02G5155</i>	649.19444-	MADS-box transcription factor 50
	00	649.20657	
	<i>TraesCS5A02G5193</i>	651.00397-	NAC domain-containing protein 86
	00	651.00821	
	<i>TraesCS7D02G5215</i>	592.53732-	Programmed cell death protein 2
	00	649.75623	
<i>qRtrl-7D</i>	<i>TraesCS7D02G5292</i>	598.08138-	Transcription factor bHLH93
	00	598.08266	
	<i>TraesCS7D02G5339</i>	601.2191-	BTB/POZ and MATH domain-containing protein 2
	00	601.22013	
	<i>TraesCS7D02G5380</i>	603.54478-	E3 ubiquitin-protein ligase ATL15
	00	603.54565	

5. Conclusions

In this study, based on exploring the ratio of RSA related traits of the 243 natural population and 123 RIL population to normal and low N supply under two N supply conditions, a total of 598 SNP markers, which were significantly associated with RSA related traits, were detected at 160 genetic loci identified by GWAS, and a total of 10 QTL loci related to RSA were discovered identified by linkage mapping. Finally, three significant RSA related QTL genetic loci, namely *qRtrl-3D*, *qRnrt-3D*, and *qRtrl-7D*, were mapped according to the responses of root architecture related traits. Which may be the new NUE related locus in wheat. These results will be helpful for marker assisted selection and further research on the cloning of NUE genes in Wheat.

Supplementary Materials: The following supporting information can be downloaded at the website of the paper posted on Preprints.org, Table S1: 243 natural population names; Table S2 Marker-trait associations for nitrogen use efficiency related traits ($P \leq 0.0001$) ; Table S3 Compare the results of previous studies.

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