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Research article

Genome-wide analysis for root and leaf architecture traits associated with drought tolerance at the seedling stage in a highly ecologically diverse wheat population

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ABSTRACT

Drought stress occurred at early growth stages in wheat affecting the following growth stages. Therefore, selecting promising drought-tolerant genotypes with highly adapted traits at the seedling stage is an important task for wheat breeders and geneticists. Few research efforts were conducted on the genetic control for droughtadaptive traits at the seedling stage in wheat. In this study, a set of 146 highly diverse spring wheat core collections representing 28 different countries was evaluated under drought stress at the seedling stage. All genotypes were exposed to drought stress for 13 days by water withholding. Leaf traits including seedling length, leaf wilting, days to wilting, leaf area, and leaf rolling were scored. Moreover, root traits such as root length, maximum width, emergence angle, tip angle, and number of roots were scored. Considerable significant genetic variation was found among all genotypes tested in these experiments. The heritability estimates ranged from 0.74 (leaf withing) to 0.99 (root tip angle). A set of nine genotypes were selected and considered drought-tolerant genotypes. Among all leaf traits, shoot length had significant correlations with all root traits under drought stress. The 146 genotypes were genotyped using the Infinium Wheat 15 K single nucleotide polymorphism (SNP) array and diversity arrays technology (DArT) marker platform. The result of genotyping revealed 12,999 SNPs and 2150 DArT markers which were used to run a genome-wide association study (GWAS). The results of GWAS revealed 169 markers associated with leaf and root traits under drought stress. Out of the 169 markers, 82 were considered major quantitative trait loci (QTL). The GWAS revealed 95 candidate genes were identified with 53 genes showing evidence for drought tolerance in wheat, while the remaining candidate genes were considered novel. No shared markers were found between leaf and root traits. The results of the study provided mapping novel markers associated with new root traits at the seedling stage. Also, the selected genotypes from different countries could be employed in future wheat breeding programs not only for improving adaptive droughttolerant traits but also for expanding genetic diversity.

1. Introduction

Wheat (Triticum aestivum L.) is one of the most important crops for

Egypt, and this can be recognized through Egypt being one of the largest wheat importers in the world to feed its vast and growing population. More than 12 million tons of wheat are imported, and by 2028, that

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Abbreviations: GWAS, (genome-wide association study); SHL, (shoot length); LA, (leaf area); SLW, (sum of leaf wilting); DTW, (days to wilting); LR, (leaf rolling); RL, (root length); RW, (root width); REA, (root emergence angle); RTA, (root tip angle); NOR, (number of roots); PS, (population structure); LD, (linkage disequilibrium).

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number is anticipated to reach more than 15 million tons. According to estimates from the Food and Agriculture Organization (FAO) of the United Nations, wheat were grown on around 3.6 million feddans (1.52 million hectares) in 2022, up from 3.5 million feddans (1.46 million hectares) the year before (https://www.fao.org/egypt/news/detail-events/en/c/1287662/).

Drought stress impacts wheat on all development stages negatively by reducing both production and quality. The effects of drought will also be worsened due to the growing world population, rising water demand for agriculture, and dwindling supplies of freshwater [1].

Because the seedling stage influences all subsequent growth phases and eventually grain output, this stage is a crucial one for drought stress [2]. As a result, wheat breeding programs urgently are needed to identify the processes behind T. aestivum's sensitivity to drought stress at the seedling stage and fully utilize natural genetic variation to improve drought tolerance in wheat genotypes. Leaf wilting, days to wilting, and stay green are important traits that were previously scored in winter and spring populations to study genetic variation in drought tolerance at the seedling stage [3,4]. Root traits at the seedling stage have received little attention. Therefore, root system architecture (RSA) provides very important information on the ability of plants to absorb water and nutrients from the soil [5]. One of the crop defense strategies to combat abiotic stress is a well-developed root system. Many root-related quantitative features, including root biomass, root length, and root volume, often displayed high heritability due to their relative stability across various conditions/environments [6,7]. Scoring leaf and root traits is essential to select the most promising drought-tolerant wheat genotypes.

Drought tolerant traits are polygenic, which makes them difficult to comprehend at the molecular and physiological levels. To genetically improve drought tolerance in wheat, strategies such as marker-assisted breeding, quantitative trait locus mapping, and introgression from the wild gene pool can be used to identify candidate genomic regions controlling drought tolerance [8]. More sophisticated approaches, including genomic selection, are now possible due to recent developments in high-throughput phenotyping and genotyping tools [9]. These approaches allow examination of the architecture of complex characteristics. To find seedling-related traits (roots and shoots) QTLs in wheat, several quantitative trait loci (QTL) analyses have been carried out to detect markers associated with drought tolerance at the seedling stage [10–12]. Root parameters including root length and root biomass were significantly influenced by numerous QTL governing plant heights and shoot dry weight, especially at the seedling stage, highlighting the critical functions of the root system in plant growth and development [5, 12-16].

The genome-wide association study (GWAS), one of the effective techniques, has been utilized to identify marker-trait associations and QTLs for target traits (e.g. drought tolerance) in wheat. GWAS has been widely employed in numerous crops, including wheat, employing genome-wide dense markers for the prediction of candidate genes [1,5, 17–19]. Sequence-based DNA markers, particularly SNP tri [8,20]. Significant SNPs were found to be mapped to potential genes involved in plant responses to abiotic stress. Large gene pools that are typical of various breeding reservoirs may be examined using high-density SNP markers for GWAS. The best method for finding robust QTLs that have an impact under both normal and stressful situations is GWAS [21-23]. The bulk of the SNPs were found at or close to the gene coding area when the identified markers were in silico searched against the International Wheat Genome Sequencing Consortium (IWGSC) reference genome. Diversity Arrays Technology (DArT) is a highly efficient genotyping approach that does not rely on the sequence availability of data [24]. DArT marker is а sequence-independent, micro-array hybridization-based marker system [24], it generates medium-density genome scans by assessing the presence versus absence of DNA fragments in genomic DNA sample representations. It may identify hundreds to thousands of polymorphic loci in a single experiment [24,25]. Sequencing of amplified inserts can thus easily provide sequence information for DArT markers of interest. Several studies have shown that this sequence knowledge can be used to assign functional significance to markers and to identify candidate genes [26–30]. Consequently, genome-wide association studies (GWAS) have become a potent and widely used for identifying alleles for the complex traits.

This work aimed to study the genetic variation in root and leaf traits under drought stress in highly diverse wheat populations, select the most drought-tolerant wheat genotypes for the breeding program, and identify candidate genes associated with root and leaf traits to be utilized in marker-assisted selection.

2. Materials & methods

2.1. Plant materials

146 spring wheat (*Triticum aestivum* L.) genotypes representing 28 different countries were used in this study. The wheat population also represented the five continents (Africa, Europe, Asia, North America, South America, and Australia) (Fig. 1a). The genotypes were obtained from Assiut University, Egypt, and Genetic Resources und Preproduction Department, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany (http://www.ipk-gatersleben.de/en/genebank/), Germany. Detailed information on the genotypes used in this study is presented in (Supplementary Table 1).

2.2. Experimental layout

Drought experiment conducted in the Cytology and Genetic Laboratory, Faculty of Science, Mansoura University, Egypt. The experiment was a randomized complete block design (RCBD) with three replications. From each genotype, four seeds were sown in each replication with a final of 12 seeds/genotype across the three replications. Seeds were sown in 6.5 \times 6 cm pots in which each pot was filled with 50 g of fertilized clay/sandy soil (1:1). Soil water capacity was estimated according to Grewal et al. [31]. The drought experiment was performed according to the protocol reported by Sallam et al. [14]. The seeds of all genotypes were handily sown in the pots and then irrigated with 20 ml (100% soil water capacity). In the second irrigation, all genotypes were irrigated with 10 ml (50% soil water capacity). During the experiment, daily humidity and temperature data were recorded. The temperature ranged from 21 - 25 °C (Fig. 1b) and air humidity (%) ranged from 50 to 55% (Fig. 1c). When all plants reached to one-leaf stage (seedling emergence), irrigation was withheld to start drought stress, and this was after seven days from the sowing date. The period of water withholding was 13 days and stopped when the leaves of \sim 70% of genotypes were fully wilted due to the effect of drought stress.

2.3. Phenotypic traits scoring

During drought treatment, some of the leaf traits were recorded. Days to wilting (DTW) as a number of days were visually from water withholding to when 50% of plants /genotypes started to wilt. Leaf wilting (LW) was also visually scored on each plant/genotype every two days, from water withholding using a scale ranging from 1 (no wilting) to 9 (fully wilted). Leaf rolling (LR) was visually scored on each plant /genotype during the experiment. The visual score of leaf holloing was extended from 1 (no leaf rolling) to 5 (leave was fully rolled).

At the end of the drought experiment, all plants of each genotype were carefully cleaned from the soil. Different leaf and root traits were scanned and measured using mage J (version1.50i) software. Root traits including root length (RL), max. width of root (RW), emergence angle of root (EA), tip angle of root (TA), and no. of root (NOR). For shoot and leaf phenotypic traits: shoot length (SHL) and leaf area (LA) were measured.

Leaf and root traits scored in this study are illustrated in Fig. 1d.

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Fig. 1. Distribution of 146 spring wheat (*Triticum aestivum* L.) genotypes on 28 different countries all over the world (a), Daily Temperature (**T**) during drought treatment experiment (b), humidity in % (**H**) during the experiment (c), visual scoring of leaf wilting (1–9) and leaf rolling (1–5) (d).

2.4. Selection for promising genotypes at seedling stage

Phenotypic selection was performed as described in Sallam et al. [14]. All genotypes were sorted from tolerance to susceptible, according to the value of the genotype, in each trait. Then, the 15 drought-tolerant genotypes were selected for each trait. The genotype was finally selected if it was among the 15 most drought-tolerant genotypes in at least four traits.

2.5. Analysis of the phenotypic traits

PLABSTAT software [32] was used to statistically estimate variance and covariance using the following statistical model:

$$Y_{ij} = \mu + g_i + r_j + gr_{ij}(error)$$

where Y_{ij} is the observation of genotype i in replication j, is the general average, g_i and r_j are the genotype and replication main effects, respectively, and the error is the interaction between genotype I and replication j. Genotypes and replications were considered as fixed and random effects, respectively. The HEI command in PLABSTAT was used to obtain broad-sense heritability (H² estimates for each trait as follows

$$H^2 = \frac{\sigma_G^2}{\sigma_G^2 + \sigma_{GR}^2}$$

where σ_G^2 is the genotypic variance and $\sigma_G^2 + \sigma_{GR}^2$ is the phenotypic variance.

The Spearman rank correlation coefficient was estimated to investigate the phenotypic correlation among all traits.

For phenological traits of wheat genotypes, descriptive statistics' mean, and standard error were calculated.

2.6. Statistical analysis of the genotypic data

2.6.1. Population structure (PS)

The PS is a statistical method for calculating relatedness correlation among individuals within a population due to mixing and historical structure, which should be carefully implemented throughout the analysis [33]. To evaluate population structure and estimate the number of subpopulations present within the target population, STRUCTURE 3.4.0 software (Pritchard et al., 2000) was used. The Burn-in period in this analysis was 100,000, with 100,000 Markov chain Monte Carlo (MCMC) replications after burn-in. The default subpopulation number (k) ranged from 1 to 10, whereas the assumed number of iterations was 3. The STRUCTURE HARVESTER software [34] was used to identify the optimal number of subpopulations (K)[35]. The principal component analysis (PCA) was also calculated for all genotypes using TASSEL v5.0.

2.6.2. Linkage disequilibrium (LD)

The LD simply refers to a nonrandom association of alleles at two or more loci[36]. TASSEL v.5.2.5 software was used to estimate linkage disequilibrium (LD) between each pair of significant markers located on the same chromosome using the squared allele frequency correlation coefficient (r2).

2.6.3. Genome-wide association study (GWAS)

Out of 146 genotypes, 111 were genotyped using two different sets of markers (Supplementary Table 2);.

- (1) 15 K Infinium SNP array (15 K set): the genotyping was done by TraitGenetics GmbH (www.traitgenetics.com). The 15 K set is an optimized version of the 90 K iSELECT chip described by Wang et al. [38]. The genotyping method revealed 12,989 SNP markers. The marker data were obtained from Tarawnsh et al. [39].
- (2) DArT profiling (DArT set): the genotyping was performed by Triticarte Pty. Ltd. (www.triticarte.com.au) which resulted in a

set of 2836 DArT markers. The marker data were obtained from Muqaddasi et al. [40].

The two sets were combined, and the total number of markers (15 K and DArT sets) was 15,825 markers. All heterozygous loci were removed, and markers were filtered based on allele frequency minimum threshold of 5% as recommended by Alqudah et al. [41]. The filtration process revealed a set of 14,045 markers that were used for conducting marker-trait association via a genome-wide association study.

The GWAS was performed by MLM, GLM, and FarmCPU. To correct the effect of population structure, PCA and kinship individually and in combination (PCA+kinship) were used in each GWAS model. The GWAS analysis was run using the Genomic Association and Prediction Integrated Tool (GAPIT) in the R environment 3.1. Significant markers were detected with a threshold P-value of 0.001 (equal to $-log10(P) \ge 3$). The Manhattan plot for each trait showing the incident markers was presented using SR plot (https://www.bioinformatics.com.cn/srplot).

In each trait, the appropriate statistical GWAS model was determined based on the results of the quantile-quantile (q-q) plot obtained from the analysis. For each significant marker, target alleles (drought-tolerant alleles) were identified based on the allele effects. The genome assembly: International Wheat Genome Sequencing Consortium (IWGSC) ensemble 2.1 was used to identify the gene annotation for each significant SNP. The position (bp) of each SNP was blasted in the ensemble plants genomic database (https://plants.ensembl.org/Triticum_ae stivum/Info/Index) and the candidate gene was identified if the significant SNP fell within the exon regions of that gene.

Linkage disequilibrium (*r*[']) was estimated between each SNP pair located on the same chromosome using TASSEL 5.0 [42] (Supplementary Table 2) and the LD heatmap was presented by SRplot website (https://www.bioinformatics.com.cn/en).

The expression of candidate genes revealed by GWAS was investigated seedling stage of wheat and under control and abiotic stress conditions based on the wheat expression database (http://www.wheat -expression.com/).

3. Results

3.1. Genotypic variation of drought-tolerance traits of wheat genotypes at seedling stage

The minimum, maximum, and the mean for each trait is presented in Table 1. Both leaf and root traits showed a huge phenotypic variation among genotypes under drought treatment at the seedling stage. For leaf traits, a wide range of genotypes was found in shoot length (6.74 to 23.54 cm (, leaf area ($0.98 - 7.96 \text{ cm}^2$), the sum of leaf wilting (17 - 32.5 days), days to wilting (2 to 8 days), and leaf rolling (1-5). For root traits, on the other hand, a wide range was found in root length (2.97 to 8.93 cm), root width (2.41 - 7.13 cm), root emergence angle ($14.28 - 46.88^\circ$), root tip angle ($18.5 - 42.74^\circ$), and no. of roots (2 - 5.25).

The variance analysis showed considerable significant differences among genotypes for leaf and root traits (Table 1). The density plot showing the distribution of all genotypes in each trait is presented in Fig. 2. High heritability estimates were found in all traits. Root traits had higher H^2 than leaf traits. The H^2 ranged in leaf traits from 0.66 (DTW) to LA (0.99), while it extended from 0.89 (NOR) to 0.99 (REA) for root traits.

3.2. Selection for most high-performance wheat genotypes under drought stress

Selected genotypes that had high performance under drought stress for leaf and root traits are presented in (Table 2). Nine genotypes; from Greece, Cyprus, the USA, Uruguay, China, and Egypt were found to be among the most drought-tolerant genotypes in at least four traits. These genotypes had low SLW and high DTW and LR. On the other hand, five genotypes from Portugal, Afghanistan, Canada, the USA, and Italy were among the most susceptible genotypes for four and/or five traits that had high SLW and low DTW and LR (Table 2).

3.3. Genotypic (r_{g}) and phenotypic (r_{p}) correlation analyses

The genotypic and phenotypic correlations among all traits are presented in (Table 3). Notably, the phenotypic correlations among traits were lower than the genotypic correlation. Concerning the correlation among leaf traits, the sum of leaf wilting (SLW) and days to wilting (DTW) had the highest significant correlation ($r_p = -0.82$ **; $r_g = -0.89$ ++). Shoot length (SHL) was highly correlated with leaf area (LA) ($r_p = 0.64$ **; $r_g = 0.65$ ++), while Days to wilting (DTW) showed a low correlation with LA ($r_p = 0.18$ *; $r_g = 0.22$ ++). Also, DTW was negatively correlated with leaf rolling (LR) ($r_p = -0.17$ *; $r_g = -0.19$ +).

Among root traits, root length (RL) showed the highest correlation with root width (RW) ($r_p = 0.62$ **; $r_g = 0.62$ ++). Root emergence angle (REA) was positively correlated with RTA ($r_p = 0.31$ **; $r_g = 0.31$ ++). Meanwhile, the number of roots (NOR) was positively correlated with RL ($r_p = 0.26$ **; $r_g = 0.29$ ++) and RW ($r_p = 0.37$ **; $r_g = 0.40$ ++). According to the phenotypic and genotypic correlation between leaf and root traits, it was observed that SHL had positive significant phenotypic correlations among all root traits. SHL had the highest correlation with RW, among root traits, with r_p of 0.39 * *. Leaf wilting traits (SLW and DTW) did not show any significant correlation with RW, RTA, and NOR.

3.4. Genome-wide association study (GWAS)

Fig. 3a illustrates the results of population structure analysis for 111 spring wheat genotypes using STRUCTURE software. The population structure was built to reveal the genetic link between genotypes within the target population and to test for the presence of subpopulations. The result of PS divided the population into two subpopulations (SP1 and SP2) (Fig. 3a). The SP1 had 55 different genotypes, while the SP2 included 56 different genotypes. The results of PS (Fig. 3b). The analysis (PCA) were in agreement with the results of PS (Fig. 3b). The

Table 1

Descriptive statistics and heritability	v estimate of leaf and root	phenotypic traits of 20-	days wheat seedlings.
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	Leaf Traits					Root Traits	Root Traits				
	SHL	LA	SLW	DTW	LR	RL	RW	REA	RTA	NOR	
Min.	6.74	0.98	17	2	1	2.97	2.41	14.28	18.5	2	
Max.	23.54	7.96	32.5	8	5	8.93	7.13	46.88	42.74	5.25	
Mean	15.51	4.13	25.31	4.43	4.6	5.42	4.84	26.89	30.06	3.98	
H^2	0.98	0.99	0.74	0.66	0.84	0.95	0.97	0.99	0.99	0.89	
F-Value	50.56 * *	221.55 * *	3.83 * *	2.91 * *	6.41 * *	19.06 * *	38.43 * *	145.49 * *	117.09 * *	8.88 * *	

Minimum, maximum, mean, F-value (among genotypes), and Heritability H2 estimates for the scored traits. SHL (shoot length), LA (leaf area), SLW (sum of leaf wilting), DTW (days to wilting), LR (leaf rolling), RL (root length), RW (root width), REA (root emergence angle), RTA (root tip angle), NOR (number of roots). * * Significant at the 0.01 level of the probability.



Fig. 2. Density diagram for all genotype in each morphological trait; (a) RW (root width), RL (root length), NOR (number of roots), LR (leaf rolling), LA(leaf area), DTW (days to wilting), (b) RTA (root tip angle), SLW (sum of leaf wilting), SHL (shoot length), REA (root emergence angle).

Table 2

The selection of the most high-performance and low-performance genotypes based on all morphological traits.

			Leaf Traits				Root Traits						
Continent	Country	Genotypes	SHL	LA	SLW	DTW	LR	RL	RW	REA	RTA	NOR	Count
high-performance genotypes													
Europe	Greece	TRI_10705	×		×	×		×			×	×	6
-	-	TRI_10707	×	×	×	×			×			×	6
Europe	Cyprus	TRI_10654	×	×		×	×						4
Europe	Cyprus	TRI_10780				×	×	×				×	4
North America	USA	TRI_11020		×	×	×	×						4
South America	Uruguay	TRI_3925		×		×	×				×		4
Asia	China	TRI_3929			×	×	×					×	4
North America	USA	TRI_3242				×	×	×	×				4
Africa	Egypt	Giza-152			×		×		×	×			4
low-performance g	enotypes												
Europe	Portugal	TRI_3564	×	×			×			×		×	5
Asia	Afghanistan	TRI_4113		×			×	×	×				4
North America	Canada	TRI_3633					×			×	×	×	4
North America	USA	TRI_4940		×	×	×						×	4
Europe	Italy	RI_4126					×	×	×			×	4

× refers that the genotype was present among the 15 high-performance genotypes in the respective traits, SHL (shoot length), LA (leaf area), SLW (sum of leaf wilting), DTW (days to wilting), LR (leaf rolling), RL (root length), RW (root width), REA (root emergence angle), RTA (root tip angle), NOR (number of roots).

Table 3
Phenotypic (normal font) and Genotypic (bold font) correlations among leaf and root traits scored

	Leaf Traits						Root Traits				
Traits	SHL	LA	SLW	DTW	LR	RL	RW	REA	RTA	NOR	
SHL	0	0.64 * *	0.11	0.01	0.04	0.26 * *	0.39 * *	0.23 * *	0.25 * *	0.24 * *	
LA	0.65 + +	0	-0.16	0.18*	0.18*	0.15	0.29 * *	0.12	0.21*	0.22 * *	
SLW	0.13 +	0.18 +	0	-0.82 * *	0.13	0.08	0.09	0.09	-0.1	0.01	
DTW	0.01	0.22 + +	-0.89 + +	0	-0.17*	-0.05	0	-0.05	0.15	0.02	
LR	0.04	0.19 + +	0.14 +	-0.19 +	0	-0.15	-0.06	0.17*	0.04	0.04	
RL	0.27 + +	0.16 +	0.07	-0.05	-0.18 + +	0	0.62 * *	-0.15	-0.07	0.26 * *	
RW	0.40 + +	0.29 + +	0.11 +	-0.01	-0.07	0.62 + +	0	-0.07	-0.1	0.37 * *	
REA	0.24 + +	0.12 +	0.11 +	-0.07	0.18 + +	-0.15 +	-0.07	0	0.31 * *	-0.02	
RTA	0.25 + +	0.21 + +	-0.12 +	0.19 +	0.05	-0.07	-0.11 +	0.31 + +	0	-0.05	
NOR	0.26 + +	0.23 + +	0.03	0.01	0.04	0.29 + +	0.40 + +	-0.02	-0.06	0	

+ , + + coefficient of correlation is larger than one and two times the standard error, respectively.

SHL (shoot length), LA (leaf area), SLW (sum of leaf wilting), DTW (days to wilting), LR (leaf rolling), RL (root length), RW (root width), REA (root emergence angle), RTA (root tip angle), NOR (number of roots).

, * * significant at the 0.05 and 0.01 level of the probability, respectively.



Fig. 3. (a) Analysis result of population structure of 111 spring wheat genotypes, (b) Principal component analysis (PCA) based on the population structure result into two sub-populations, (c) Principal component analysis (PCA) for genotypes based on their continent.

PCA was also performed based on the continent and it was found that genotypes from the same continent were randomly distributed in the two clusters (Fig. 3c).

The LD (*r2*) was estimated among the significant SNPs on the same chromosome to determine whether they represented the same QTL or individual QTLs. The LD between each pair of the significant markers located on the same chromosome for each trait is shown in detail in Supplementary Table 2. Chromosome 3B had the largest genomic region that included seven significant markers associated with LR followed by a genomic region, on 1D chr., consisting of six significant markers associated with SL Significant markers that are associated with leaf rolling trait show high LD on 1B, 2D,3B, and 5 A chromosomes. Also, High LD genomic regions were found on 1B, 1D, 5 A, 5D, and 5D chromosomes which related to no. of roots, shoot length, root length, days to wilting, and root emergence angle traits respectively showed high LD values.

A summary of GWAS results is presented in Table 4 and a detailed analysis is presented in (Supplementary Table 2). A total of 208 QTLs were found to be associated with leaf and root traits under drought stress. All comparative QQ plots are represented in (Supplementary Figures 1 from a to j) and Manhattan plots for leaf and root traits are presented in (Supplementary Figures 2a and b). The number of QTL detected for leaf traits (132)was higher than those associated with root traits (76) Genome B had the highest number of QTLs with 89 followed by genome A (77) and genome D (32) The percentage of significant markers on each genome is represented in Fig. 4a. For leaf traits, the distribution of QTLs on the chromosomes is presented in Fig. 4b. The QTLs associated with leaf traits were divided into 17 DArT and 115 SNP markers. Among the nine models, Farm-CPU+PCA was the best GWAS model for all leaf traits based on the q-q plot results. The highest number of QTLs was found to be located on 1 A (14) while the 4B chromosome had the lowest number of QTLs (one). Leaf rolling (LR) had the highest number of QTL [37], whilst, SL had the lowest number of QTL (16) Out of the 132 QTLs, 69 had major effects with an R² of > 10%. It was noted that LR had the highest number of major QTLs (26)with a range extending from 10.4 to 19.23%.

On the other hand, 11 and 65 DArT and SNP markers were significantly associated with root traits, respectively. The q-q results indicated that the FarmCPU+PCA model was the best model that fitted with RL, RW, and REA, while MLM+PCA was suitable for RTA and NOR. The 3B and 7B had the highest number of QTLs with a total of 12, while, one QTL was found to be located on 1D, 2 A, 3 A, and 7D (Fig. 4c). The number of QTLs for root traits ranged from six for RTA to 20 for both RL and RW. A set of 48 QTL was found to have major effects with R² ranging from 10.03 to 17.21%. Each root trait had QTLs with major and minor effects except RTA which all significant markers associated with this trait had major effects (R² = 12.3–17.21%).

Notably, markers associated with more than one trait were detected (Table 5 and Fig. 5). For leaf traits, seven markers (six SNP and one

Table 4

Summary of significant markers associated with morphological traits under drought stress at a suggestive P-value.

Trait Leaf Traits	Model	No. of markers	p-value range	R2 (%) range	No. Candide gene
DTW	PCA- FarmCPU	28	1.98E-05: 0.0009	7.112: 14.28%	22
SLW	PCA - FarmCPU	32	2.32E-05: 0.0009	8.174: 14.275%	23
SHL	PCA - FARMCPU	16	1.66111E-05: 0.0009	8.356: 14.328%	9
LA	PCA - FarmCPU	19	3.01593E-05: 0.0009	7.408: 14.726%	9
LR	PCA - FarmCPU	37	9.11E-07:.0009	7.159: 19.231%	26
Root Traits					
RL	PCA - FarmCPU	20	9.05559E-05: 0.0009	8.333: 13.578%	9
RW	PCA - FarmCPU	20	9.06383E-05: 0.0008	8.211: 13.213%	15
REA	PCA - FarmCPU	19	0.0001: 0.0009	8.912: 14.454%	14
RTA	PCA - MLM	6	3.88E-05: 0.0008	12.331: 17.215%	5
NOR	PCA - MLM	8	0.0003: 0.0009	9.524: 14.879%	4

DTW (days to wilting), SLW (sum of leaf wilting), SHL (shoot length), LA (leaf area), LR (leaf rolling), RL (root length), RW (root width), REA (root emergence angle), RTA (root tip angle), NOR (number of roots).



Fig. 4. The percentage of significant markers on each genome (a), Number of QTLs of leaf traits on each chromosome (b), Number of QTLs of root traits on each chromosome (c).

Table	5
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Common markers associated with more than one trait under drought stress at *P-value* > 0.001 and suggestive *p-value*.

Marker name	Chr.	Pos.	Traits	Candidate Gene	Protein Coding
Excalibur_c77035_156	1 A	1 A:49252812-49252907	DTW, SLW	TraesCS1A02G067200	Pentatricopeptide repeat
wPt-669577	1 A	1A:547955524-547955698	DTW, SLW		
Kukri_c5497_312	2 A	2 A:38914240-38914320	DTW, SLW	TraesCS2A02G084800	PADRE domain
BobWhite_c41535_52	2B	2B:58979394-58979494	DTW, SLW	TraesCS2B02G099600	DnaJ domain
wsnp_Ex_c21092_30220342	2B	2B:58978366-58978566	DTW, SLW	TraesCS2B02G099600	DnaJ domain
CAP8_c359_95	3 A	3 A:74364950-74365042	DTW, SLW	TraesCS3A02G108700	F-box domain
BS00049997_51	5B	5B:626069850-626069950	DTW, SLW	TraesCS5B02G453200	RNA recognition motif domain
Excalibur_c8883_1144	7 A	7 A:730429650-730429741	RL, RW	TraesCS7A02G557600	Sucrose synthase
CAP12_c1816_325	7B	7B:21668948-21669048	RL, RW	TraesCS7B02G022400	F-box domain
RAC875_c5744_412	7B	7B:740055517-740055617	RL, RW	TraesCS7B02G482400	P-loop containing nucleoside triphosphate hydrolase
Tdurum_contig76289_1530	7B	7B:587911890-587911990	RW, REA	TraesCS7B02G332200	Protein of unknown function

(Chr.) chromosome on which the marker was detected, (Pos.) position of the marker on wheat chromosome, days to wilting (DTW), sum of leaf wilting (S_LW), root length (RL), root width (RW), root emergence angle (REA).

DArT marker) were significantly associated with DTW and SLW. The wPt-669577 marker located on 1 A had major effects on DTW (10.9%) and SLW (11.9%). For root traits, three shared markers were detected for RL and RW, and one shared marker was found for RW and REA. Only one SNP marker (Excalibur_c8883_1144, 7B) had major effects on RL and RW. No shared markers were found between leaf and root traits (Fig. 6a).

3.5. Candidate genes associated with leaf and root traits

Each significant marker was blasted in the Ensemble Plants genomic database to identify candidate genes and their protein-coding. A set of 77 different gene models were detected for leaf traits, while 40 candidate genes were found for root traits (Fig. 6b, Supplementary Table 2). One shared gene model *TraesCS6B02G322300* located on the 6B chromosome was found to be associated with LA (leaf traits) and RW (root traits). The protein-coding for each candidate gene was investigated. The 77 candidate genes associated with leaf traits encode 65 different proteins, while 40 genes associated with root traits encode 29 different between. Six proteins were common between root and leaf traits.Out of 117 gene models, 60 were previously published with evidence for their association with drought tolerance in wheat (Supplementary Table 2). The expression of all gene models was investigated in the leaves and roots at the seeding stage under non and abiotic stress tolerance (Fig. 7, Supplementary Table 3). A set of 20 gene models were not tested before

at the wheat seedling stage. The expression of gene models associated with leaf traits and root traits are presented in (Fig. 7a and b), respectively.

The gene model and their coding protein for the common markers are presented in Table 5. All common markers were located within gene models except wPt-669577 (DArT marker). All gene models encode functional proteins except TraesCS7B02G332200 (RW and REA) which encodes to protein with an unknown function. In leaf traits (DTW and SLW), two SNPs were located within the same gene model *TraesCS2B02G099600*. The expression of these genes indicated that 8 genes were found to be up-regulated in the shoots and roots, while only one gene TraesCS3A02G108700 was found to be down-regulated in wheat seedlings under abiotic stress (Supplementary Table 3).

4. Discussion

4.1. Genetic variation in drought tolerance

Under the present climate change scenario, breeding for drought tolerance needs to receive substantially more focus and funding. Drought stress affects wheat crops at all development stages. The complexities of drought tolerance reflect the fact that the effect of the stress is dependent on the developmental stage of the plant, and the intensity of the stress can fluctuate over time [39]. For all leaf and root traits, the analysis of variance revealed high and considerable significant variation



Fig. 5. Physical position of the pleiotropic markers on each chromosome.



Fig. 6. Markers associated with root traits(RT) and leaf traits(LT) detected by GWAS(a), and shared genes and encoding protein (EG) between RT and LR) (b), gene expression of the common gene associated with RT and LT; TPM(transcript per million).

among genotypes. The visual scoring scale for SLW, DTW, and LR provided quick and dependable methods for assessing drought tolerance in the whole population with minimal time and effort [14,43]. The heritability of these traits was high in this study and all earlier studies [14, 43]. Therefore, these traits can be quickly and accurately used for screening a large number of genotypes at the seedling stage [43]. Comparing this screening technique to field circumstances, where there are many confounding environmental factors , it was proven to be a valid way to analyze the variances in root systems. This study examined various spring wheat seedling phenotyping features under drought

stress. The high heritability estimates indicated that the selection based on these traits is feasible for the genetic improvement of drought tolerance at the seedling stage is feasible.

Leaf wilting is an important trait because it indicates a lack of moisture in the soil, leading to a deficiency of water absorption and transmission to the shoots [44]. Earlier studies scored the leaf wilting trait one time to measure the impact of drought stress on the plant such as Zhou et al. [45] in Soybean and Sayed et al. [46] in spring barley. However, sequential scoring of leaf wilting (SLW) during the experiment provided a precise assessment of drought tolerance compared to single



Fig. 7. The candidate genes expression of leaf traits in leaves/shoots tissue at the seedling stage under different conditions (control, drought and PEG) (a), The candidate genes expression of root traits in leaves/shoots tissue at the seedling stage under different conditions (control, drought and PEG) (b). Golden star refers to the genes which association with more than one trait.

LW scoring because it recorded the effect of drought stress on the leaves regularly during drought experiments [2]. All LW values were summed together resulting in one trait that reflected the symptoms of drought stress, which is more accurate than LW as it represents the effect of drought during the entire period. Days to wilting provided important information on the time (in days) for each genotype to respond to the water shortage. Many crops, including Solanum Lycopersicon and Ipomoea batatas, have had substantial research on the relationship between drought tolerance and DTW [47,48]. Leaf rolling is a desirable trait in wheat that can reduce transpiration and increase dry matter accumulation [49]. Large specialized adaxial epidermal cells (bulliform cells) responsible for leaf rolling additionally improve wheat plant's wettability. As a result, the leaf rolling dynamics of a wheat plant support the high input use efficiency that can mitigate production losses during drought stress and enable efficient photosynthetic activity in leaves [50]. Leaf rolling is caused due to the change in the water potential within the epidermal and bulliform cells. So, high leaf rolling indicates a high probability of drought tolerance. Under drought stress, a wheat plant exhibits early maturity, a reduction in plant growth, and a reduction in leaf area for water equilibrium by the root system and plant tissues [51]. Root angles are also a very useful trait for drought studies. Manschadi et al. [52] confirmed the role of root angle in drought tolerance as they reported a close relationship between root system architecture and the angle of seminal root growth at the seedling stage. Wheat adapted to low rainfall regions had narrower seminal root angles and deeper root systems, while in higher rainfall they have wider seminal root angles, which presumably facilitate water and nutrients from a larger subsurface area [53]. The high genetic variation found in all leaf and root traits reported in this study can be utilized to select the most promising genotypes that can be used for future breeding programs to improve drought tolerance in wheat at this critical stage.

4.2. Phenotypic selection for drought tolerance

All genotypes in each trait were sorted based on the direction of drought tolerance (from tolerant to susceptible) to phenotypically select the promising drought-tolerant genotypes [3]. Instead of single-trait selection, multiple-trait selection is used to identify the tolerant and susceptible genotypes under drought conditions which provides an effective way for selection [14]. This will help the wheat researchers to precisely select the desirable genotypes to be utilized as candidate parents in wheat future breeding programs. The selected nine drought-tolerant genotypes were from different countries, indicating the usefulness of crossing among them to produce promising drought-tolerance cultivars and expanding the circle of genetic diversity in wheat. Interestingly, two genotypes TRI_10705 and TRI_10707 were among the most tolerant genotypes in six traits including leaf and root traits. These two genotypes were highly tolerant to drought stress compared to the other genotypes in this study. Therefore, these genotypes are highly recommended to be crossed in future breeding programs for improving drought tolerance in spring wheat.

4.3. Genotypic and phenotypic correlations

Understanding the relationship between leaf and root traits contributed to comprehending multiple mechanisms related to drought resistance in seedlings. The highly significant genotypic and phenotypic correlations among all drought-stressed features aided in the understanding of the various mechanisms connected to drought tolerance in the seedling stage. The genotypic correlation was shown to be greater than the phenotypic correlation in all traits such as those described by Ahmed et al. [43] indicating that the parameters may be controlled by common QTL. The high negative correlation results between SLW and DTW found in this study were similar to those previously published by Ahmed et al. [43] in a highly diverse spring wheat population, Sallam et al. [14] in a biparental winter wheat population, and Sallam et al. [43] in diverse winter wheat population at seedling stage under drought stress. This demonstrated that these two traits are highly correlated across wheat populations and in both spring and winter types. Therefore, breeders can save time and effort by scoring only one of these two traits in selection programs to improve drought tolerance. Notably, shoot length had a positive and significant correlation with all root traits. The relationship between shoot length and drought tolerance was highlighted before by Ahmed et al. [43] and Sallam et al. [14] who scored shoot length before applying drought stress in wheat at the seedling stage. They found a highly positive significant correlation

between shorter plants and drought tolerance. Bearing in mind that shoot length in this study was scored after applying drought stress. So, the deeper root system can enhance shoot length during drought stress. Leaf rolling is an essential drought tolerance mechanism with polygenic regulation and additive effects that have been observed in wheat, rice, maize, and sorghum, however, only a few studies on leaf rolling in hexaploid wheat have been reported [54]. Rolling of leaves helps a water-stressed plant to reduce exposed leaf area while also reducing transpiration and gas exchange through the stomata [55]. In this study, LR had a low correlation with both shoot and root traits except with leaf area and days to wilting which means that leaf rolling is related to leaf area and consequently plays an important role in drought resistance. A wide root system would improve its ability to acquire nutrients and support plant growth, root traits such as root tip angle and root emergence angle are highly correlated and that's referred to both traits had the same mechanism [56]. Significant correlations among root system size, root width, and the number of roots and different root traits were previously reported by Xie et al. [56].

4.4. Genome-wide association mapping for leaf and root traits

The result of this study revealed very important markers associated with leaf and root traits under drought stress at the seedling stage. Very few studies reported markers associated with seedling traits. For leaf traits, SNP markers associated with DTW and SLW were reported before in winter wheat at the seedling stage under drought stress in diverse populations and biparental populations [15]. Moreover, DArT markers associated with SLW and DTW were previously reported in a spring wheat core collection at the seedling stage under drought stress by Ahmed et al. [43]. The position and location of DArT markers associated with DTW and SLW detected in this study were compared with those reported by Ahmed et al. [43] and no shared markers were found. Although Sallam et al. [14] used different types of SNP (genotyping-by-sequencing), the position in pb between the significant SNPs detected in their study and SNPs detected in this study were compared. All significant SNPs detected in both studies were located in different genomic regions on the same chromosome. Additionally, significant SNPs located on the 1 A chromosome (14 SNPs) were reported in this study for leaf traits but, Sallam et al. [14] did not find any significant SNP location on 1 A in the diverse population and one SNP on 1 A in the biparental population. The non-shared markers or position (physical distance) for the same traits reported in different studies supported the notion that drought tolerance is a very complex genetic trait controlled by many genes. Also, important-novel significant SNPs were reported for LA and LR in this study. Many QTLs were reported for the LA trait at the reproductive growth stage [57] but none was reported at the seedling stage under drought stress in earlier studies. At the seedling stage under drought stress, Yang et al. [58] found an important genomic region on the 7 A chromosome between 708-721 Mb that controlled different degrees of leaf rolling. In our study, three SNPs for LR were found to be located on 7 A and they were located on different positions. Unfortunately, very few studies reported QTL associated with LR and LA under drought stress at the seedling stage. Therefore, our results shed light on the genetic architecture of these two traits by mapping 19 and 37 SNPs for LA and LR, respectively.

Promising significant SNPs were reported in this study for root traits. Genome-wide association mapping was performed before to reveal the genetic architecture of wheat roots under drought stress in wheat [5]. A major QTL for root architecture located on 6 A in wheat seedlings under drought stress was reported by [59]. In this study, no significant SNP for root traits was found in that chromosome.

Most of the significant DArT and SNP markers had major effects with $R^2 > 10\%$. The number of QTLs with major effects (116) was higher than those with minor effects ((89) Quantitative traits are controlled by a few genes with major effects and many genes with minor effects. Major QTLs are more important for facilitating the potential of genetic or QTL

mapping [60]. However, it is very important to identify both effects to understand the genetic control of target traits. Moreover, the reason for detecting many major QTLs in this study is the use of ImageJ software which is considered one of the high-throughput phenotyping technologies that provide precise phenotyping [60]. This precise phenotyping that links to genotypic data in association analyses will facilitate the identification of a specific set of QTLs for complex traits [60]. The ImageJ was previously used to identify significant SNPs associated with target traits (e.g., root angle in wheat) in QTL and GWAS studies No shared markers were found between leaf and root traits [53,61–63].

The flanking sequence around each significant SNP was blasted to identify candidate genes associated with leaf and root traits. Most of the candidate genes detected by GWAS in this study were previously reported with their functions under drought stress (Supplementary Table 2). Many genes detected by GWAS were found to be expressed under natural drought stress or/and under polyethylene glycol (PEG) treatment. This indicated the usefulness of GWAS to identify the truly significant associations. Moreover, the non-expression genes (no expression data available) indicated that these genes could have a relation with drought tolerance in wheat. Therefore, it is worth testing the expression of genes under drought stress in wheat to validate their biological function. One of the advantages of using GWAS is to also identify new alleles and genes for important target traits such as drought tolerance.

Although there were no shared markers between leaf and root traits, one candidate gene TraesCS6B02G322300 (KUP6) was found to have SNPs for LA (wsnp_Ku_c15761_24469459) and two RW (wsnp_Ku_c15761_24469519). The physical position of the flanking sequence including the target SNP is 570977423-570977623 bp for wsnp_Ku_c15761_24469459 and 70977483-570977683 bp for wsnp_Ku_c15761_24469519 with a distance of 60 bp between them. The TraesCS6B02G322300 (KUP6) encodes Potassium Transporter which was previously reported with its role in enhancing drought tolerance in rice, barley, and Arabidopsis [64,65]. Potassium plays a vital role in the plant adaption mechanism under drought stress as the greater the amount of K⁺ uptake provides a higher level of drought tolerance [66]. This gene was also found to have a higher expression under PEG treatment and natural drought stress than in non-stress conditions in the leaves and shoots at the seedling stage (Fig. 6c). By looking at the protein levels, nine coding and functional shared proteins between leaf and root traits. Some of these proteins such as: (F-box-like domain superfamily, Protein kinase-like domain superfamily, Alcohol dehydrogenase, N-terminal, Potassium transporter, EamA domain, and Protein of unknown function DUF707) have evidence of their relation to drought stress (https://kne tminer.com/cereals/).

Investigating the LD structure allows for the discovery of genomic areas that have large genetic effects on complex trait expression, as well as the determination of the marker density required to correlate genotypes with traits[67]. The high and low LD discovered throughout the three genomes provide a high possibility of locating target QTL with large and minor impacts in the existing materials[68]. High LDs were identified between significant markers on the same chromosome, indicating that these markers are frequently co-inherited[69]. One marker from each high LD genomic region can be selected and transformed into a kompetitive allele-specific PCR (KASP) marker for further validation in a different genetic context[70,71].

4.5. Promising pleiotropic markers for improving drought tolerance at the seedling stage

Interestingly, shared markers among leaf traits and among root traits were found in this study (Table 5, Fig. 5). These markers had pleiotropic effects. For leaf traits, seven markers were found to be associated with DTW and SLW. These results were expected due to the high correlation between these two traits. High LD was found between the wsnp_Ex_c21092_30220342 and BobWhite_c41535_52 located on 2B

chromosome, indicating that these two markers are likely to be coinherited and represent the same QTL (Supplementary Figure 3). The other markers located on the same chromosome can be considered individual QTL with pleiotropic effects. No significant markers of LD were found among the three markers located on the 7B chromosome for root traits. These markers shed light on their usefulness in marker-assisted selection. The effect of these markers should be investigated in different genetic backgrounds under drought stress before being used in MAS for improved drought tolerance at the seedling stage in wheat [72]. These SNPs were located within eight candidate genes. For leaf traits, the Excalibur_c77035_156 marker was located within TraesC-S1A02G067200 and encodes Pentatricopeptide repeat. Pentatricopeptide repeat (PPR) genes play an important role in regulating mitochondrial RNA editing and the PPR is considered a potential gene resource in the improvement of drought tolerance in rice [73]. Interestingly, the two SNPs located on 2B were located within TraesCS2B02G099600 which encodes the DnaJ domain. In Arabidopsis, it was reported that the DanJ gene plays an important role in proline biosynthesis, increasing drought tolerance [14] [74]. F-box domain (TraesCS3A02G108700) positively regulated drought tolerance, likely because they maintain intracellular homeostasis via a significant increase in positive regulatory factors content of drought stress [75]. TraesCS5B02G453200 encodes the RNA recognition motif domain which plays a vital role in regulating tolerance to drought and salt stresses in plants.

For root traits, sucrose synthase is encoded by TraesCS7A02G557600 (Excalibur_c8883_1144 marker) [76]. Sucrose synthase is among the key enzymes involved in sucrose metabolism [77]. Earlier studies have reported significant increases in the activity of sucrose synthase activities under dehydration and osmotic stress [78]. Notably, the Tdur-um_contig76289_1530 marker was found to be located within TraesCS7B02G332200 which encodes an unknown functional protein. The expression of this gene could be validated using real-time PCR as this protein might play an important role in drought tolerance.

These markers having pleiotropic effects are very useful and they can be converted to the KASP markers for further validation under drought stress in other wheat populations to be used in MAS for the genetic improvement of drought tolerance at the seedling stage in wheat.

5. Conclusions

Leaf and root traits associated with drought tolerance provided essential information on various mechanisms that can be used by juvenile wheat plants to alleviate the effect of drought stress at the seedling stage. Among all leaf traits, SL was found to be significantly associated with root architecture traits. The phenotypic correlation and GWAS results revealed that leaf and root traits might be controlled by different mechanisms, therefore, selection for drought tolerance genotypes at the seedling stage is highly recommended to include root and leaf traits. In this regard, a list of nine highly diverse wheat genotypes was selected. Moreover, novel SNPs associated with new leaf and root traits (especially LR, RW, REA, and RTA) under drought stress at the seedling stage such as were reported in this study for the first time. The results of this study can be used to improve drought tolerance in spring wheat at early growth stages.

Informed Consent Statement

Not applicable.

CRediT authorship contribution statement

R.A.A performed phenotyping and genotyping experiments, analyzed the data, and wrote the manuscript; Y.M.H. and M.M.E. supervised phenotyping data and edited the manuscript; A.B. provided the plant material, helped in discussing the results, and drafted the paper; A.

S. designed the whole study, supervised all phenotypic and genotypic analyses, and edited the manuscript. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this page.

Data availability statement

All data generated or analyzed during this study are included in this published article and its supplementary information files.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2024.01.020.

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