Genome-wide SNP discovery, identification of QTLs and candidate genes associated with morpho-physiological and yield related traits for drought tolerance in maize

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Abstract

Drought is one of most important abiotic stresses affecting maize yield. The present study was aimed to map genomic regions associated with the morpho-physiological and yield related traits in maize under well-watered and water-deficit stress conditions using recombinant inbred population (RILs) derived from the cross HKI161 × SNJ201126 by genome-wide SNP discovery using genotyping-by-sequencing approach. Phenotyping of RILs showed normal frequency distribution for most of the traits indicating their polygenic nature. The SNP linkage map was generated using 1241 SNP markers which were distributed over 10 linkage groups and covered a total genetic distance of 5471.55cM. A total of 40 QTLs were identified for the various morpho-physiological and yield related traits under well-watered (26) and water-deficit stress conditions (14). Of these 14 were major QTLs, 26 were minor QTLs and two QTLs (*qCW2-1, qCH1-1)* were detected under both conditions. Three QTL hotspots regions were identified, one for net CO_2 assimilation rate (AN) and transpiration rate (TR) (*qAN3-1, qTR3-1* and *qAN3-2*) on chromosome (chr) 3, cob height (CH) and plant height (PH) (*qCH8-1* and *qPH8-1*) on chr 8 and cob weight (CW), total biomass (TB) and grain yield (GY) (*qCW2-1*, *qTB2-1*, *qTB2-2* and *qGY2-1*) on chr 2 capturing phenotypic variability ranging between 10.10–22.33%. The QTL hotspot region for AN & TR encoded for genes involved in signal transduction pathway under water-deficit stress tolerance, maintenance of plant membrane and cell wall integrity and cross membrane transport. While QTL hotspot for PH and CH encoded genes involved in carbon utilization in plant growth and development, directed movement of proteins in the cell and transmembrane transporter activity. The QTL hotspot for yield encoded genes involved in signal transduction and plant development. These regions can be further fine mapped and used for marker assisted breeding in maize.

Introduction

Drought stress is one of the major abiotic stresses affecting productivity of maize worldwide. It causes major yield losses when occurring at anthesis-silking interval and at grain filling stages[1]. Thus, improving drought tolerance has been the major goal of many breeding programs. Drought tolerance is a complex trait controlled by many genes[2] with high genotype × environment interactions. Dissection of component secondary traits (morpho-physiological and yield related) can be useful in improving tolerance to drought stress[3,4]. Although conventional breeding led to the improvement of grain yield in maize, the genetic gain was mostly reported for favourable environment. The genetic enhancement for drought stress conditions is still very limited. Understanding plant's response to drought stress at the genomic level are critical for improving tolerance to drought[5]. It is important to unravel molecular mechanisms in response to drought stress in maize, identify quantitative trait loci (QTLs) and candidate genes for accelerating genetic gain through marker-assisted selection[6,7,8]. Drought stress induces a number of physiological and biochemical changes like reduction in leaf water content, photosynthesis and alterations in metabolism in plants. These effects are manifested through reduced plant height, cob weight, biomass and yield[9]. The next generation sequencing technologies (NGS) are being widely used through linkage and association mapping in identification of QTLs for complex traits like drought tolerance which forms the basis for marker-assisted selection (MAS) breeding. For QTL identification, the most commonly used population are bi-parental mapping population such as recombinant inbred lines (RILs) where the genotypes with contrasting traits are selected, crossed and employed in population development. QTL mapping allows the identification of chromosomal fragments linked with the trait of interest. Earlier PCR based markers (RFLP, RAPDs and SSRs) were used for generation of genetic linkage map. Due to the rapid development of sequencing techniques, more efficient markers like single nucleotide polymorphisms (SNPs) are now frequently being used for development of high-resolution maps[10].

Recent advances in genomics has unravelled the genetic dissection of important agronomic and physiological traits. A number of quantitative trait loci (QTLs) for drought stress tolerance have been employed in a number of studies in maize[1,11,12,13,14,15]. QTL mapping studies for identification of QTLs under both well-watered and water-stress conditions can be categorized as 'constitutive'[16] while QTLs detected only in specific environment conditions as 'adaptive'[12]. The co-localized QTLs for morpho- physiological and yield related traits under stress helps us to validate whether a particular trait is constitutive / adaptive and its role in improving field level drought tolerance. With the availability of whole genome sequence data of maize (B73 reference genome), mining of candidate genes responsible for the phenotypic variation could help in understanding the molecular mechanism of drought tolerance in maize. Studies on candidate gene identification using linkage mapping and association mapping are reported for many important traits[1,17].

Therefore, the present study was aimed to map genomic regions associated with the drought tolerance related morpho-physiological and yield traits using maize RILs by genotyping through high throughput SNP sequencing approaches. Also, identification of both major and minor effect QTLs and their associated candidate genes was carried out.

Results

Phenotyping for morpho-physiological, yield and its related traits:

The RIL population showed wide variation for the various morpho-physiological traits and yield related traits such as relative water content (RWC), normalized difference vegetation index (NDVI), canopy temperature depression (CTD), quantum yield (fv/fm), net CO_2 assimilation rate (AN), stomatal conductance to water vapor (g_s), transpiration rate (TR), leaf temperature (LT), anthesis-silking interval (ASI), plant height (PH), cob height (CH), cob length (CL), number of kernel rows (NKR), number of kernels per row (NKPR), cob weight (CW), total biomass (TB) and grain yield / plant (GY) under well-watered (WW) and water-deficit (WD) stress conditions (Supplementary Table S1a, b). All the traits were affected by water-deficit stress. Frequency distribution histograms of morpho-physiological and yield traits under WW and WD stress conditions were given in Fig. 1. All these traits showed near normal distribution except quantum yield which was negatively skewed under water-deficit stress conditions while stomatal conductance was positively skewed under well-watered conditions. Based on the significance values obtained using Kolmogorov–Smirnov test the traits CTD, NDVI, AN, LT, PH, CH, CL, NKPR, CW, GY and TB showed normal distribution under both well-watered and water-deficit stress conditions while the trait g_s and TR showed normal distribution under WD stress conditions (Supplementary Table S2). This indicates that selected RILs captured the genetic variability of whole mapping population to be utilized for QTL identification for these traits. The descriptive statistics of mapping population under WW and WD stress for morpho-physiological and yield related traits including

coefficient of variation (CV%), skewness, kurtosis and heritability were given in Supplementary Table S3. Moderate broad-sense heritability (H^2) was observed for all the traits with a range of 0.45 – 0.72 in the population. A wide range of coefficient of variation was observed among all the traits.

The pooled ANOVA of trials across seasons (*rainy and post rainy*) showed significant interaction between seasons, treatment and genotype for most of the traits. For traits RWC, NKPR and TB, all interaction effects were significant except season × treatment (Supplementary Table S4). For the trait ASI, the interactions effect for treatment × genotype and season × treatment × genotype was not significant. The significant genotype, environment and their interaction variances for traits indicated that these traits were influenced by both genetic and non-genetic factors. Simple correlation coefficient analysis revealed significant positive correlations between NDVI and PH, CH, CW, GY and TB, AN and g_s, TR, g_s and TR, PH with CH and CL, CH and TB, CL and NKR, NKPR, GY and TB, NKR and NKPR, CW with GY and TB, GY with TB under both well-watered and water-deficit stress conditions. Similarly, significant positive correlations between QY and g_s, TR, LT and TB under WD stress conditions was observed (Supplementary Table S5).

QTL mapping using high throughput SNP genotyping

The number of raw SNPs, polymorphic SNPs between the parents after filtering with MAF ≤ 0.05% and their distribution in various chromosomes, number of mapped SNPs in linkage map and average marker interval was presented in Table 1. The maximum number of markers (219) were detected on chromosome (chr) 2 and minimum number of markers (65) on chr 10. A total of 40 QTLs were associated with traits analysed, of which 26 were detected under well-watered and 14 under water- deficit stress conditions. Among these 14 major QTLs capturing ≥15% phenotypic variation were identified for the traits viz., RWC, NDVI, CTD, AN, g_s, TR, PH, CH, CL, NKPR and CW. The LOD score values for these QTLs varied between 2.54 and 6.07 with phenotypic variation captured varied between 15.05 to 22.34% (Table 2). While, 26 minor effect QTLs capturing <15% phenotypic variation were identified for the traits viz., AN, ASI, CH, CTD, g_s, GY, LT, NDVI, NKPR, NKR, QY, TB and TR. The LOD score values for these QTLs ranged between 2.52 and 3.37 with PVE% ranging between 6.75 to 14.91 (Supplementary Table S6). One major QTL for the trait RWC was detected on chromosome 9 with a LOD score of 2.71 and capturing 16.3% PVE. For the trait NDVI one major and minor QTL were detected on chr 2 having LOD scores of 3.93, 3.19 and capturing PVE % of 18.59, 14.02 respectively. Two minor QTLs were detected for the trait QY on chr 1 and 7 with a LOD score of 2.86, 2.81 and PVE% of 14.62, 14.47 respectively. For net CO_2 assimilation rate (AN), a major and a minor QTL were detected on chr 3 (LOD score of 3.24, 2.52) capturing 17.24, 14.25 PVE% respectively. For the trait stomatal conductance to water vapor two major QTLs were detected on chromosome 6 and three minor QTLs one on chr 3 and two on chr 7. The LOD ranged from 2.54 to 4.45 and PVE% ranged from 9.69 to 18.21. For the trait transpiration rate one major QTL was detected on chromosome 7 (LOD: 6.07; PVE%: 21.47) and two minor QTLs one each on chr 1(LOD: 2.87; PVE%: 10.81) and 3 (LOD:2.72; PVE%: 10.11).

Among yield traits, one major QTL was detected for plant height on chr 8 (LOD score of 3.19 and PVE% of 17.98). For cob height one major QTL and two minor QTLs were detected on chromosome 1 with a LOD score of 3.49, 2.52, 2.90 and PVE% of 15.05, 13.84, 11.68 respectively. For total biomass, three minor QTLs, two on chr 2 and one chr 5 were detected with a LOD score of 2.53, 2.58, 2.55 and PVE% of 13.51, 11.50, 13.04 respectively. For cob weight one major QTL was detected on chr 2 under both WW and WD stress conditions. For grain yield two minor QTLs one each on chr 2 and 6 were detected (LOD score of 2.78, 2.54) capturing PVE% of 10.54, 11.33 respectively.

Identification of co-localized and QTL hotspot region

Out of the 40 QTLs, three co-localized QTLs were identified. For traits TR and ASI two QTLs (*qTR1-1*, *qASI1-1*) were co-localized at the marker interval rs128441140 - rs128842621 on chr 1 with LOD score of 2.87, 2.61 and capturing phenotypic variation of 10.81, 13.92 % respectively, while, g_s and GY QTLs (*qg$_s$6-2* and *qGY-6-1*) were co-localized at the marker interval S6_89546385 - S6_179562549 on chr 6 with LOD score of 2.54 and capturing phenotypic variation of 15.86, 11.77% respectively. For the traits g_s and TR, QTLs (*qg$_s$7-1*, *qTR7-1*) were co-localized at the marker interval S7_166501967 - rs130671858 on chr 7 with LOD score of 2.73, 6.07 and capturing phenotypic variation of 11.3, 21.07 % respectively.

In the present study, we have also identified three QTL hotspots regions. The marker interval, S3_169283017- rs129386882 (15cM) on chromosome 3 encompassed three QTLs for the traits net CO_2 assimilation rate and transpiration rate *qAN3-1*, *qTR3-1* and *qAN3-2* capturing 10.10 to 17.24 % phenotypic variation (Fig. 2). While, the marker interval, rs130916094 - rs849450935 on chromosome 8 (27cM) encompassed two QTLs for cob height and plant height *qCH8-1* and *qPH8-1* with LOD scores 2.78, 3.19 and the PVE% 14.9, 17.98% respectively (Fig. 3). The region between the marker interval, S2_213060766 - S2_14679066 on chromosome 2 (33cM) encompassed three QTLs for the traits cob weight, total biomass and grain yield *qCW2-1*, *qTB2-1*, *qTB2-2* and *qGY2-1* having LOD scores of 2.53 to 4.23 and capturing 10.54 to 22.33% phenotypic variation (Fig. 4).

Epistatic interaction among QTLs

Seventeen significant digenic epistatic QTLs for traits RWC, QY, AN, gs, TR, LT, ASI, CL, CW and TB were detected (Table 3; Supplementary Fig. S1a, b). The LOD score values ranged between 5.0 to 5.91 while the PVE% ranged between 11.42 – 37.43%. The negative epistatic values (add by add) indicated higher epistatic effect of the recombinant genotype than the parental genotype. The epistatic effect values of QTLs for traits TB, AN, RWC, CL, LT, and QY were negative while for traits g_s, QY, ASI, TR, CL, AN and CW was positive. The genomic region on chromosome 2 between the marker interval of rs129243511-S2_229825946 showed epistatic interaction for CW on chromosome 8, located between the marker interval of S8_145945904 - S8_148216407 and this interaction contributed to 37.43% of phenotypic variation. The genomic region on chromosome 7 between the marker interval of rs131600615 - S7_50455390 showed epistatic interaction for RWC on chromosome 9, located between the marker S9_41587672 - rs131737404 contributing 33.03% of phenotypic variation. Two epistatic interaction were identified for AN. First the on chromosome 1 between marker S1_3798004 - rs128441140 showed epistatic interaction with region on chromosome 3, located between rs129555629 - S3_183844216. This interaction contributed to 17.60 % of phenotypic variation. Second, on chromosome 2 between markers, rs727228961- rs276685886 showed epistatic interaction for AN with chromosome 3, located between interval of S3_2276048 - S3_89193637 which contributed to 16.71% of phenotypic variation. The genomic region on chromosome 2 between the marker interval of

rs131971876 -rs129196105 showed epistatic interaction for TR with the region on chromosome 7, located between S7_131791490 - S7_136261108 and contributed to 29.55% of phenotypic variation.

QTL-by-Environment Interaction Analysis

Using the MET (multi-environmental trials) module of ICIM a total of 78 QTLs were identified for different traits (Supplementary Table S7). Of these 32 QTLs were common to those identified by ICIM-ADD method. One QTL each for traits g_s, ASI, GY and two QTLs for trait LT which were identified by ICIM-ADD method but were not detected by MET analysis. The MET analysis identified 46 QTLs for traits viz., RWC, QY, AN, CH, ASI, TB, GY, NDVI, LT, CW and PH which were not detected by ICIM-ADD method. The phenotypic variation captured by additive and dominance effects [PVE (A)] varied from 3.42-14.36 % and the PVE (A by E) (additive and dominance by environment effects for corresponding QTLs) varied from 0 - 7.92 %. The PVE (A by E) was significantly lower than PVE (A). For traits AN, g_s, NDVI, NKPR, QY and TR significant differences were observed in the QTL × E interaction effect namely, the LOD (AbyE), PVE (AbyE) indicating the significant effect of the environment (Table 4).

Identification of candidate genes in the genomic region spanning QTLs

The major and minor QTLs associated with morpho-physiological and yield related traits, their chromosome position, locus ID, start and end position, SNP type and position, size of the locus, genes and their biological / molecular function was given in Table 5, Supplementary Table S8. The number of genes identified for the QTLs of various traits ranged between 1-3. The genes encoded for different traits belonged to the categories signal transduction (RWC - *Zm00001eb395310* - Guanine nucleotide exchange factor SPIKE 1; GY - *Zm00001eb297570* - Protein-serine/threonine phosphatase), transcription factors (NDVI - *Zm00001eb118010* - G2-like-transcription factor 27; CTD - *Zm00001eb325260* - AP2-EREBP-transcription factor 201; ASI - *Zm00001eb295810* - NAC type transcription factor (NAC87), transporter activity (AN - *Zm00001eb146040* - Chloride channel protein; g_s - *Zm00001eb324180* - Sugar carrier protein C; TR - *Zm00001eb015510* - Phospholipid-transporting ATPase; CH - *Zm00001eb363270* - Calcium-transporting ATPase; NKPR - *Zm00001eb076560* - GDP-mannose transporter (GONST5), cell wall biosynthesis and organization (TR - *Zm00001eb144960* - Lipoxygenase; TR - *Zm00001eb145080* - Pectin acetylesterase), photosynthesis (g_s, TR -*Zm00001eb324240* - Chlorophyll a-b binding protein, chloroplastic) and Carbon utilization (PH - *Zm00001eb359190* - Carbonic anhydrase; CH - *Zm00001eb002270* - GlyceraIdehyde phosphate dehydrogenase B1).

Discussion

Field phenotyping of RIL population for various morpho-physiological and yield related traits under well-watered and water-deficit stress conditions showed high variability for RWC, NDVI, CTD, QY, AN, g_s, TR, LT, ASI, PH, CH, CL, NKR, NKPR, CW, TB and GY indicating segregation of these traits. The RIL population also showed transgressive segregation for RWC, AN, PH, CH, TB and GY under WW and WD stress conditions. Frequency distribution for most of these traits revealed near normal distributions indicating its polygenic nature and potential for genetic enhancement. Positive and negative correlations were observed among various traits. Positive correlations were observed between net CO_2 assimilation rate and its related traits such as g_s and TR and between yield and its related traits such as CW, TB and GY under both WW and WD stress conditions. The trait NDVI showed positive correlation with PH, CH, CW, TB and GY both under WW and WD stress conditions. Transpiration rate showed positive correlation with g_s, AN with g_s and TR under WD stress conditions. While significant negative correlation was observed between RWC with NDVI and LT. In a similar study, positive correlations between NDVI and PH with grain yield were reported across populations and treatments[18]. In the present study, it is interesting that significant positive correlations were observed between physiological and yield traits, while few traits showed significant negative correlations under WW conditions (Supplementary Table S5). In an earlier study[19] no significant correlations were observed between physiological and yield related traits although significant correlations existed among themselves.

SNP genotyping and QTL identification

In this study, the linkage map consisted of 1241 SNP markers distributed over 10 linkage groups, covering a total genetic distance of 5471.55 cM with an average marker density of 4.4 per cM. RIL mapping populations are widely used for QTL identification[20,21]. In the present study, 40 QTLs were identified for various physiological and yield related traits under well-watered (26) and water-deficit stress conditions (14). Of these 14 were major QTLs and 26 were minor QTLs. In the present study, one major QTL for RWC under WW conditions was identified on chr 9. QTLs for RWC were reported on all the chromosomes except 7, 8 and 9[19] while, in another study QTLs for RWC on chr 9 and 10 near the markers *bnl14.28a* and *csu48* respectively were reported[11]. In our study, one major and one minor QTL for NDVI under WD stress conditions were detected on chromosome 2 (*bin 2.10*). In earlier study, 18 QTLs for NDVI and plant height in two $BC_1F_{2:3}$ backcross populations (LPSpop and DTPpop) was reported[18]. QTLs for NDVI influencing the trait stay green (*SEN6*) were also detected in *bins 8.01* and *2.07* in the DTPpop[18]. We have also identified two minor QTLs for QY under WW conditions on chr 1 and 3 and one each major and minor QTLs for the trait CTD under WW conditions on chr 7 and 1 respectively.

For the trait net CO_2 assimilation rate, one each major and minor QTL on chr 3 (bin 3.05) under WW conditions were identified. This region also showed the presence of one minor QTL for TR under WW conditions. QTLs for TR were also detected on chr 1 and 7 respectively. For the trait g_s two major QTLs were identified on chromosome 6 (WD) and one each minor QTLs on chromosome 3 (WD) and 7 (WW). In an earlier study[22] 32 QTLs were detected associated with chlorophyll-a content, chlorophyll-b content, total chlorophyll content, net CO_2 photosynthetic rate, stomatal conductance, intercellular CO_2 concentration and transpiration rate in F_2 mapping populations. In addition, only one mapping study for photosynthetic performance in maize have been reported under drought environments[23]. Nineteen major QTLs controlling different physiological traits were identified in another study[24] under drought-stressed and well-watered regimes. For stomatal conductance QTLs have been detected on all chromosomes except chr 5 in maize[11,25]. We have also detected two minor QTLs for LT (WW) on chr 6 and 8 and for ASI 3 minor QTLs, on chr 1 (WW, WD) and on chr 6 (WW). For ASI, a total of 33 QTL were reported under stressed environments distributed over all the chromosomes[26]. QTLs for ASI under well-watered and water stressed conditions were also identified by[27].

In the present study, one major QTL for plant height on chr 8 (*bin 8.06*) under WW conditions was identified. One major (WD) and two minor QTLs (WW, WD) on chr 1 and one each minor QTL on chr 3 (WD) and chr 8 (WW) respectively for cob height were also identified. For plant height and ear height a number of QTLs have been identified on all the 10 chromosomes[28,29,30]. Twenty-one QTLs were identified for PH and EH in three common genomic regions in the two bi-parental populations under multiple environments[30]. A QTL *qHT_YZ5a* was identified[31] located at *bin 5.04–5.06* which was co-localized with QTLs previously identified[30,32,33,34]. Another QTL for PH, (*qPH_Y1a*/ *qEH_Y1a*) on chr 1 which encoded for the gene *ZmRPH1* was also identified[35]. This gene was known to be linked to plant height and cob height which was also reported in another study[31]. But the major and minor QTLs (*qCH1-2, qCH1-1*) identified on chr 1 in our study were localized at a different location and were novel. While QTLs for PH and EH on chromosomes 1, 3 and 8 were reported earlier as well[36]. Since PH and EH are significantly correlated in maize breeding populations, some QTL for these traits are mapped in the same genomic regions[37]. In our study as well, QTLs for both PH and CH, *qPH8-1-1*, *qCH8-1* were located in the same genomic region (*bin 8.06*). However, some QTLs were also mapped at different locations suggesting that the genetic mechanism of PH and EH may be similar, but not identical.

Grain yield is a highly complex, quantitative trait controlled by numerous genes with small effects[38]. In the present study, one major QTL for CL was identified on chr 2 under WW conditions. Previous reports have identified around 171 QTLs for CL of which 19 were localized on chr 2[39]. For the trait NKR one minor QTL on chr 2 under WW conditions was identified. For the trait NKPR major QTLs were identified on chr 2 and chr 10 under WW conditions in this study. A total of 97 QTLs were identified for the trait NKPR and 8, 10 QTLs were located on chr 2 and 10 respectively. A QTL atlas with major genes for grain yield and its component traits were reported earlier[39]. QTL for kernel number encoding for glutamine synthetase isoenzymes (*Gln1–3*) (*Zm00001d017958*) were identified by[40]. In the present study one QTL hotspot on chr 2 was identified for CW, TB and GY. In our study QTLs for CW and GY on chr 2 were physically located at 241494911 -241497812 bp, 14676667-14679369bp respectively and differed from the previously reported meta QTLs[39]. One more QTL for grain yield on chromosome 6 located at 179555644-179566050bp also differed from the previously reported meta QTLs on this chromosome and may be novel. Three minor QTLs for total biomass, two on chr 2 and one on chr 5 under WW conditions were identified in the present study. In a similar study, seven coincident QTLs were identified[41] for two traits biomass production and leaf area in the interval *bnlg1832-P2M8/j* (*bin 1.05*) on Chr 1. In another study, QTL for yield on chr 1 was found co-locating with the QTLs for root traits, total biomass, and osmotic potential in a region of about 15cM[42].

The missing proportion of phenotypic variance for any trait may be partly explained by epistasis[43]. Epistasis is a non-allelic interaction changing the degree of phenotypic expression by suppressing/enhancing the expression of interacting genes[44, 45]. In the present study, the variation in the physiological and yield related traits i.e., RWC, QY, AN, g_s, TR, LT, ASI, CL, CW and TB may be influenced by a few major loci plus several minor loci and epistatic effects. The evaluation of the population in multiple locations, year, treatments such as temperature, different watering regimes will help us in studying the stability of QTL across the environments[46]. Therefore, joint analysis over years and locations can be used to establish the stability of QTLs and to estimate interaction between QTL and environment. In the present study, traits AN, g_s, NDVI, NKPR, QY and TR showed significant effect of environment on expression the QTL.

In-silico analysis of the genomic region having QTLs and identifying candidate genes

In-silico analysis of the genomic region encompassing the QTLs helped in identifying candidate genes imparting stress tolerance. Two genes *Zm00001eb395070* and *Zm00001eb395310* were found to be associated with RWC and had SNP strongly associated in the QTL region on chr 9 respectively (Table 5). The gene *Zm00001eb395070* encodes WEB family protein which plays an important role in chloroplast avoidance movement so as to avoid strong light to preserve the photosynthetic machinery[47]. The gene *Zm00001eb395310* encodes *guanine nucleotide exchange factor SPIKE 1*, which has a function in small GTPase mediated signal transduction and is known to contribute towards cytoskeletal reorganization required for cell shape and tissue development[48]. The gene encoding *G2-like-transcription factor 27* (*glk27*) was associated with NDVI and showed a strong associated SNP for NDVI on chromosome 2. The gene glk27 belongs to *Myb* transcription factors with an established relationship to chloroplast development and negative regulation of leaf senescence[49]. It was proposed as a second golden producing factor and identified as controlling cellular differentiation in maize leaves.

Two genes *Zm00001eb033440* and *Zm00001eb033450* were associated with CTD and were linked with SNP in the QTL region on chromosome 1 encoding cupredoxin superfamily protein and RNA-binding protein33c respectively. The QTLs located on chr 7 encodes for two genes *Zm00001eb325260* annotating *AP2-EREBP-transcription factor 201* while *Zm00001eb325270* encoding *ARM repeat superfamily protein*. Two genes *Zm00001eb001130* and *Zm00001eb001140* were associated with QY and harbouring one each strongly associated SNP in the QTL region on chromosome 1 and encoded for F-box protein and dihydrodipicolinate reductase respectively. The gene *Zm00001eb119130* associated with QY on chr 3 encodes for plasma membrane-associated cation-binding protein 1. The QTLs for ASI, *qASI1-2*, on chr 1 encoded for transcription factors *bHLH-transcription factor 35* (*Zm00001eb002830*) and *LOB transcription factor* (*Zm00001eb003910*), while *qASI6-1* on chr 6 encoded for *NAC type transcription factor* (*NAC87*) (*Zm00001eb295810*).

Carbon metabolism is very crucial for the overall plant growth and development. In the plant functional area of gas exchange and carboxylation, three major QTLs one each for AN, g_s, TR were found on chromosome 3, 6 and 7 respectively. On the other hand, 5 minor QTLs one each for AN, g_s and TR on chr 3 while, one for g_s on chr 7 and one for TR on chr 1. Five and nine genes were located on major and minor QTLs respectively.

With regard to the QTL hotspot for traits AN and TR, the major QTL (*qAN3-2*) on chromosome 3 harboured a gene *Zm00001eb146040* which encodes a chloride channel protein. This protein is a proton coupled chloride transporter and acts as an outwardly directed proton pump linked to amino acid decarboxylation[50] with a key role in hormonal signalling under abiotic stress. While the minor QTL (*qAN3-1*) encompassed 3 genes. The gene *Zm00001eb144000* encodes E3 ubiquitin-protein ligase RGLG1 which functions as a positive regulator of ABA signalling[51] and as a negative regulator of drought stress response[52]. The gene *Zm00001eb144010* encodes for amino acid/auxin permease20 with a known function in amino acid transport[53]. While the gene *Zm00001eb145030* encodes BTB/POZ and TAZ domain-containing protein 3 with a key role in ubiquitin conjugation pathway[54]. These genes were important in signal transduction pathways and transporter activity in imparting water-deficit stress tolerance. The minor QTL for TR, *qTR3-1* encoded for two

genes. Gene *Zm00001eb145080* encoded for pectin acetylesterase with a key role in cell wall organization[55], while gene *Zm00001eb144960* encoded for lipoxygenase with a functionality involved in lipid metabolism and contributes to the responses to biotic and abiotic stress as well as senescence[56]. These genes were associated with maintenance of plant membrane and cell wall integrity (Fig. 2).

Two genes *Zm00001eb280280* and *Zm00001eb278890* were located on chromosome 6 associated with major QTL for g_s. The gene *Zm00001eb280280* which encodes for *brassinosteroid insensitive 1-associated receptor kinase 1* is involved in signal transduction pathways, positively regulates the BR dependent plant growth pathway[57]. While, the gene *Zm00001eb278890* encodes transcription elongation factor SPT5 with a key function in regulation of transcription[58]. For the trait g_s, a minor QTL region on chr 3 having a gene *Zm00001eb120960* which encodes for *putative transcription factor bHLH041* with a function in regulation of transcription[59]. Also, two genes *Zm00001eb324180* and *Zm00001eb324240* were found to be located in the minor QTL region for g_s on chromosome 7. The gene *Zm00001eb324180* encodes for sugar carrier protein C with a function in monosaccharide transmembrane transporter activity[60]. The gene *Zm00001eb324240* encodes for chlorophyll a-b binding protein, chloroplastic with a key role in photosynthesis especially light harvesting in photosystem I and chlorophyll binding[61]. This same region of minor QTL for g_s on chr 7 was also found to be associated with a major QTL for TR. Another minor QTL on chr 1 encompassed the gene *Zm00001eb015510* encoding for phospholipid-transporting ATPase which is involved in phospholipid translocation[62]. While, two genes *Zm00001eb295640* and *Zm00001eb295670* were found to be associated with LT and having a strong association with SNP in the QTL region on chr 6 encoding rop guanine nucleotide exchange factor 9 and putative leucine-rich repeat transmembrane protein kinase family protein respectively. The QTL located on chr 8 encoded for the gene *Zm00001eb368460* coding for outer membrane OMP85 family protein.

In the QTL hotspot region for PH and CH on chr 8, two genes *Zm00001eb360480* and *Zm00001eb359190* were linked with the PH. the gene *Zm00001eb360480* encodes for glutamate synthase 1 [NADH] chloroplastic involved in glutamate biosynthesis[63,64]. The gene *Zm00001eb359190* encodes carbonic anhydrase which has the primary role in carbon utilization in plant growth and development[65]. Earlier genes related to PH were identified to the various biosynthetic pathways such as hormone synthesis, transport and signalling[66]. While CH *QTL* encompassed two genes namely gene *Zm00001eb364820* encoded for AP-4 complex subunit epsilon involved in the directed movement of proteins in the cell. While, the gene *Zm00001eb363270* encoded for calcium-transporting ATPase with a cation transmembrane transporter activity (Fig. 3).

The minor QTL for CH on chr 1, was associated with the gene *Zm00001eb011970* encoding LIM zinc-binding domain-containing protein DA1-2 related with a function in ubiquitin binding which regulates the expression of cell cycle genes and contributes to long distance phloem transport. While, another minor QTL on chr 3 harboured gene *Zm00001eb151120* encoding pentatricopeptide repeat-containing protein which is targeted to chloroplast or mitochondria binds organellar transcripts and regulates their expression eventually with profound effects on plant development under environmental stresses[67]. The gene *Zm00001eb151330* encodes receptor-like serine/threonine-protein kinase involved in the reversible phosphorylation which is one of the most important protein post translational modification. The genes encoded by QTLs *qNKR4-1*, *qNKPR2-1* and *qNKPR10-1* were mostly involved in signal transduction and transcription factors while QTL *qNKPR2-2* encoded for genes involved in transporter activity.

In the QTL hotspot for yield on chr 2, cob weight QTL which was detected both under WW and WD stress conditions encompassed the gene *Zm00001eb117750* encoding proline-rich receptor-like protein kinase PERK4. This gene is involved in protein serine/threonine kinase activity required during ABA mediated activation of Ca^{2+} channels and regulates ABA signalling pathway[68] that plays a critical role in drought stress mediated signal transduction. The TB minor QTLs in this region *qTB2-1,* harboured gene *Zm00001eb107320*, encoding for organic cation/carnitine transporter 7 with a key function in ion transport and ATP binding. While *qTB2-2* and *qGY2-1,* encompassed the gene *Zm00001eb072580* encoding *OSJNBb0016D16.16-like* protein which is uncharacterized (Fig. 4). The TB minor QTL on chr 5 harboured two genes of which, the gene *Zm00001eb221070* encoded for tetratricopeptide repeat (TPR)-like superfamily protein and gene *Zm00001eb239390* for meiosis 5 involved in cell division. Minor QTL for GY was located on chr 6 encompassed two genes, the gene *Zm00001eb297570* encoded protein-serine/threonine phosphatase and gene *Zm00001eb297580* encoded for pentatricopeptide repeat-containing protein mitochondrial.

Conclusion

The present investigation revealed high variation for various physiological and yield related traits in the mapping population. The present study identified major and minor QTLs related to the morpho-physiological and yield related traits under well-watered and water-deficit stress conditions. The QTLs identified in this study were novel as the genomic locations of the QTLs identified differed from the previous studies. Co – localization of the QTLs for traits g_s and GY on chr 6 (*qg_s6-2*, *qGY6-1*), g_s and TR on chr 7 (*qg_s7-1*, *qTR7-1*) were detected. QTL hotspots for AN and TR on chr 3, (*qAN3-1*, *qTR3-1* and *qAN3-2*) and PH and CH on chr 8 (*qCH8-1*, *qPH8-1*) and traits CW, TB and GY on chr 2, (*qCW2-1*, qTB2-1, *qTB2-2*, qGY2-1) were detected. The function of the major candidate genes associated with abiotic stress tolerance QTLs (AN: chloride channel protein; PH: carbonic anhydrase; CW: Proline-rich receptor-like protein kinase; g_s, TR: chlorophyll a-b binding protein) corroborated with the trait of interest. The QTL regions identified in this study could be introgressed into elite varieties through marker assisted breeding. The putative candidate genes can be isolated and functionally characterized. The high yielding and better performing RILs can be used for genetic enhancement in maize.

Materials And Methods

Plant material

Contrasting genotypes were identified for drought tolerance based on evaluation over years for drought related morpho-physiological traits[69]. The drought tolerant SNJ201126 and susceptible HKI161 genotypes were crossed and subsequently advanced to F_9 generation following single cob method for

developing RILs mapping population. During rainy season 2014, initial bi-parental cross was made between tolerant and susceptible genotypes. Subsequently F_1 generation was self-pollinated for nine generations up to F_9 to develop a RIL mapping population consisting of 264 single plant progenies.

Phenotyping for morpho-physiological and yield related traits

Morpho-physiological characterization of the mapping population (264 lines) along with parents was carried out in *rainy* season 2018 and *post-rainy* season 2018-19. The genotypes were sown in randomised complete block design (RCBD) with three replications at a single row plot of 2.5 m having 60 cm between rows and 25 cm plant spacing. The whole population was grown under two water regimes i.e., well-watered (WW), in which plants were grown under irrigation and water-deficit (WD) stress where irrigation was given only up to vegetative stage i.e. 45 days after sowing (DAS). The experiment was maintained with appropriate plant protection measures and recommended package of practices to raise a healthy crop. During 2018 *rainy* season the weekly average temperature varied between 17.9 ^{o}C to 31.3 ^{o}C with relative humidity from 57.1 – 84.5%, While during the *post rainy* season, temperature recorded was 11.4 – 30.4 ^{o}C with relative humidity between 40.9–83%. The total rainfall received was 377mm and 16 mm during *rainy* and *post rainy* seasons respectively (Supplementary Fig. S2).

Water-deficit stress was imposed at anthesis-silking initiation (ASI) stage with a dry spell of 10 days. The various parameters viz., relative water content (RWC), normalized difference vegetation index (NDVI), canopy temperature depression (CTD), quantum yield (fv/fm), net CO_2 assimilation rate (AN), stomatal conductance to water vapor (g_s), transpiration rate (TR), leaf temperature (LT), anthesis-silking interval (ASI), were recorded both under well-watered and water-deficit stress conditions. Yield contributing traits viz., plant height (PH), cob height (CH), cob length (CL), number of kernel rows (NKR), number of kernels per row (NKPR), cob weight (CW), total biomass (TB) and grain yield / plant (GY) were recorded on three representative plants of each genotype. Relative water content (RWC) was measured using formula RWC (%) = [(FW – DW) / (TW – DW)] × 100, where FW – fresh leaf weight, TW – turgid leaf weight after rehydration, DW – the dry leaf weight after oven drying[70]. Normalized difference vegetation index (NDVI) was measured using GreenSeeker 505 (Manuel NTech Industries Inc., Ukiah, CA, USA). This measures the reflected light on the canopy of crops at 660 nm (red) to 770 nm (near-infrared) bands. The measure of NDVI at a certain point of the image at a particular phenophases of the crop is equal to the difference in the intensities of reflected light in the red and infrared range divided by the sum of these intensities. A handheld infrared thermometer (Crop TRAK, Spectrum Technologies, Inc.) was used to measure canopy temperature between 11.30 am to 12.30 pm. Canopy temperature depression was calculated by the formula ambient temperature – canopy temperature. Quantum yield, a measure of photosystem efficiency was measured by using FluorPen FP 100 (Photon Systems Instruments, Czech Republic). Net CO_2 assimilation rate, stomatal conductance to water vapor, leaf temperature and transpiration rate were recorded by LICOR LI-6400 photosynthesis instrument (LICOR instruments, Inc. Lincoln, USA).

SNP genotyping

Cluster analysis was done to group the RIL mapping population into different groups by using combined mean data of morpho-physiological traits under well-watered and water- deficit stress conditions. The RILs population were grouped into 8 clusters having similarity within cluster. For SNP genotyping, a subset of 79 RILs were selected from these 8 clusters representing the entire genetic diversity of the mapping population, along with parents SNJ201126 and HKI161 in triplicates. SNP genotyping was carried out at Bionivid Technology Pvt. Ltd. Bangalore, India. The Illumina NGS workflow for SNP genotyping was as follows. The young leaves of 15 days old seedlings of each genotype were used for DNA isolation using DNAeasy plant mini kit (Qiagen). The DNA quality and quantity were checked by agarose gel electrophoresis and Nanodrop respectively. For library construction, DNA was fragmented randomly and adapters were ligated to the 5’ and 3’ ends. These fragments were then amplified by PCR and purified from the gel. Clusters were generated by loading the library into a flow cell, where fragments were captured on a lawn of surface-bound oligos complementary to the library adapters. After cluster generation the templates were sequenced. Sequencing was carried out using Illumina SBS technology that detects single bases as they are incorporated into template strands. As all 4 reversible terminator-bound dNTPs were present during each sequencing cycle, natural competition minimizes incorporation bias and greatly reduces raw error rates as compared to other technologies. This resulted in a highly accurate base-by-base sequencing that virtually eliminates sequence-context-specific errors, even within repetitive sequence regions and homopolymers. Sequencing data was then converted into raw data for analysis. The Illumina sequencer generates raw images utilizing sequencing control software for system control and base calling through an integrated primary analysis software called RTA (Real Time Analysis). The BCL (base calls) binary is converted into FASTQ utilizing Illumina package bcl2fastq. The total number of bases, reads, GC (%), Q20 (%), and Q30 (%) were calculated for all the samples.

Statistical Analysis

The individual and combined seasons data was used for statistical analysis. Frequency distribution histograms for all traits were generated using Matplotlib program tools. Matplotlib is a cross-platform, data visualization and graphical plotting library for Python. Pyplot module was used to generate plots while 'Scipy.stats' module used to compute and draw the histogram of control and stress data which has a large number of probability distributions and created evenly spaced points over a specified interval on X axis using 'numpy.linspace' and displayed the norm of probability density function. Test for normality was done following Kolmogorov–Smirnov method[71]. Descriptive statistics was calculated using SAS version 9.3 (SAS Institute Inc., Cary, NC, USA). Individual and pooled combined analysis of variance (ANOVA) of two seasons morpho-physiological data, Pearson’s simple correlation and heritability estimates under study were done using SAS version 9.3. For pooled analysis, the homogeneity of variance was tested using Bartlett’s test[72]. Broad sense heritability was calculated as per the following formula

Broad sense heritability ($(\mathrm{H}^2) = \frac{\sigma^2 G}{\sigma^2 P}$ where $\sigma^2 G$ is the total genotypic variance and $\sigma^2 P$ is the total phenotypic variance.

Bioinformatics analysis

SNP calling and filtering

Raw reads of sequencing data were generated in FASTQ format for all samples and imported into TASSEL GBS pipeline[73,74] implemented in TASSEL Version 5.0. Zea mays, B73, Zm-B73-REFERENCE-NAM-5.0 - Genome - Assembly – NCBI (https://www.ncbi.nlm.nih.gov/assembly/GCF_902167145.1/) was used as the reference genome. The sequence reads qualifying filtering steps were mapped onto the genome using Burrows-Wheeler Alignment (BWA) tool[75]. The mapped reads were then exported as Sequence Alignment Map (SAM) file for SNP calling and genotyping[76]. A total of 176 Gb data was generated for the whole samples sequenced. To filter the parent's call, replicates were first merged by ensuring that at least two replicates had observed calls and the most common allele was taken as the parent call, the alternate call within replicates of parents reflects genotyping errors. Further, SNPs with minor allele frequency (MAF) $\leq$ 0.05% were filtered out before analysis.

Linkage map construction and QTL analysis

Linkage map construction and QTL analysis was carried out using QTL IciMapping software, version 4.2[77]. SNP data of DNA bases (i.e. A, T, G or C) was converted into the format recognized in QTL IciMapping (i.e. A, B, or H) software using the SNP conversion functionality. SNPs showing non-polymorphism in parents or progenies, or missing in one or more parents were deleted by this functionality. Linkage map construction was carried out using MAP functionality and had three steps: grouping, ordering and rippling. Grouping was based on anchored marker information and a threshold of LOD score of 2.5 for unanchored markers. The ordering algorithm used was K-optimality by recombination, number of random nearest neighbouring (NN) route (10). Criteria used in rippling are Window size 5 and by REC. Finally outputting generates the linkage map. The anchoring and genotypic data generated along with the phenotypic data was used for QTL identification. A total of 1241 SNPs were finally selected for analysis which was distributed in 10 linkage groups covering a total genetic distance of 5471.55 cM.

Using the BIP functionality, (mapping of additive (including dominance, if any) and digenic epistasis genes) these SNPs were studied for their association with morpho-physiological and yield related traits. The mapping method used was inclusive composite interval mapping method (ICIM) for QTL with additive (and dominance) effects (ICIM-ADD). The parameters used were deletion of missing phenotypic data, mapping parameters were stepwise scanning by 1cM, phenotype on marker variables (PIN) 0.001, and a LOD threshold of 2.5. QTL effects by log-likelihood ratio (LOD), additive effect of identified loci and phenotypic variation explained (PVE%) were estimated. Those QTLs showing PVE% ≥15% were termed as major effect and <15% as minor effect QTLs. The standard procedure was followed for QTL nomenclature[78] while QTL hotspot was determined following methods described by[79] with minor modifications. The QTL hotspots were searched in a sliding window size of 20 cM and regions with two or more co-localizing QTLs was identified. An epistatic analysis was performed by the IciMappingVer.4.1 EPI epistatic module, and default parameter settings were used (LOD = 5, step = 1 cM, and stepwise regression probability < 0.0001).

The combined phenotyping data of two seasons i.e. *rainy* and *post rainy* seasons in two different environments well-watered and water-deficit stress was used to carry out the combined QTL analysis with additive-by-environment (A by E) interactive effects in a MET (multi-environment trial) module of ICIM method[77]. The parameters of QTL analysis were set as LOD = 2.5, permutations =1000, step= 1 cM and PIN = 0.001. The CI of each QTL was determined by LOD>3.

Functional annotation of SNPs and identification of candidate genes

For the functional annotation of the selected SNPs, variant effect predictor tool in Ensembl plants (https://plants.ensembl.org/Zea_mays/Tools/VEP) was used. VEF tool analyses the variants and predicts the functional consequences of known and unknown variations. The reference genome assembly was Zm-B73-REFERENCE-NAM-5.0. For each SNP rs-ID, location, allele, consequence, gene, feature type, feature, biotype, exon/intron, TREMBL protein IDs were obtained. The details of the genes associated were further studied using the maize genome database (https://www.maizegdb.org) and putative functions of candidate gene associated with the traits were identified.

Declarations

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Author contributions

B.S. Carried out the parent selection, crossing and developing RILs. Statistical analysis, manuscript writing, editing and critically reviewed the manuscript; Y.V. Field phenotyping of the parents, crossing program, RIL evaluation, QTL mapping and drafted the manuscript; M.V. Physiological characterization of population, N.R. Assisted in data analysis; M.P. Infrastructural facilities, manuscript editing; N.J field experiment, S.K.Y. Field experiments, reviewed the manuscript; M.M. Manuscript editing and reviewed the manuscript. V.K.S. manuscript editing. All authors reviewed the manuscript.

Competing interests

The authors declare no competing interests.

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Tables

Table 1. Numbers of SNPs on 10 chromosomes of maize used for QTL mapping.

	Chr1	Chr2	Chr3	Chr4	Chr5	Chr6	Chr7	Chr8	Chr9	Chr10	Total
Raw SNPs	7145	5539	4997	4853	5246	3879	3846	4306	3438	3247	46496
Polymorphic SNPs between the parents after filtering with MAF ≤ 0.05%	1878	1409	1419	1171	1302	1030	1188	1171	842	851	12261
Mapped SNPs in linkage map	144	219	182	85	96	95	163	119	73	65	1241
Average marker interval (cM)	5.61	3.14	3.86	5.84	5.77	4.15	3.49	4.12	4.97	6.23	

Chr: Chromosome; MAF: Minor allele frequency; cM: centi Morgan

Table 2. Major QTLs identified for various morpho-physiological and yield related traits using combined phenotyping data of both *rainy* and *post rainy* seasons under well-watered and water-deficit stress conditions.

QTL name	Treatment	Chr	Position of the QTL	Left Marker	Right Marker	LOD	PVE (%)	Add	Interval map distance (cM)
qRWC9-1	WW	9	46	S9_136739244	S9_137996982	2.71	16.3	0.91	43.5 - 46.5
qNDVI2-2	WD	2	388	rs131350195	S2_66658066	3.93	18.59	0.03	378.5 - 388.5
qCTD7-1	WW	7	413	S7_153755512	rs130675358	4.21	19.52	-0.23	404.5 - 414.5
qAN3-2	WW	3	324	S3_174361072	rs129386882	3.24	17.24	-2.6	322.5 - 327.5
qgs6-1	WD	6	109	S6_126753475	rs836167502	4.45	18.21	-0.02	105.5 - 112.5
qgs6-2	WW	6	271	S6_89546385	S6_179562549	2.54	15.86	0.03	252.5 - 282.5
qTR7-1	WW	7	162	S7_166501967	rs130671858	6.07	21.47	0.78	160.5 - 162.5
qPH8-1	WW	8	200	S8_155152093	rs849450935	3.19	17.98	-6.24	195.5 - 204.5
qCH1-2	WD	1	740	S1_6365045	rs818095140	3.49	15.05	4.16	738.5 - 741.5
qCL2-1	WW	2	13	S2_4548124	S2_2226727	2.77	15.94	-0.65	2.5 - 23.5
qNKPR2-2	WW	2	559	S2_27769950	rs131322326	5.37	15.34	1.49	558.5 - 559.5
qNKPR10-1	WW	10	404	S10_137217074	S10_140006671	5.1	15.75	-1.51	399.5 - 405
qCW2-1	WW, WD	2	442	S2_213060766	S2_18995588	3.03	17.95	8.59	434.5 - 445.5

RWC: Relative water content; NDVI: Normalized difference vegetation index; CTD: Canopy temperature depression; AN: Net CO_2 assimilation rate; g_s: Stomatal conductance to water vapor;

TR: Transpiration rate; PH: Plant height; CH: Cob height; CL: Cob length; NKPR: Number of kernels per row; CW: Cob weight; WW: Well-watered; WD: Water-deficit stress; Chr: Chromosome;

LOD: Logarithm of odds ratio; PVE (%): Total phenotypic variance in percentage explained by the QTL

Table 3. Epistatic interactions observed for various morpho-physiological and yield related traits.

Trait Name	Treatment	First Chr	Position1 (cM)	Left Marker1	Right Marker1	Second Chr	Position2 (cM)	Left Marker2	Right Marker2	LOD	PVE (%)
RWC	WD	7	545	rs131600615	S7_50455390	9	255	S9_41587672	rs131737404	5.01	33.03
QY	WW	1	270	S1_38965206	rs724789387	4	125	rs129634115	rs277328423	5.66	20.44
QY	WD	3	100	S3_1736455	S3_2771091	10	145	S10_151061996	rs132618945	5.03	11.42
AN	WW	1	115	S1_3798004	rs128441140	3	350	rs129555629	S3_183844216	5.08	17.60
AN	WW	2	365	rs727228961	rs276685886	3	635	S3_2276048	S3_89193637	5.91	16.71
g_s	WW	6	40	S6_177038002	S6_148279548	7	120	rs130642102	rs836234066	5.32	16.60
g_s	WW	7	270	S7_11679195	rs130488903	8	250	rs814260212	S8_73973624	5.48	18.05
TR	WD	2	265	rs131971876	rs129196105	7	365	S7_131791490	S7_136261108	5.79	29.55
LT	WD	4	60	S4_166565742	S4_182462050	6	170	rs55624911	rs130311785	5.22	34.85
ASI	WD	2	640	S2_221752549	rs833320055	3	270	rs839843727	rs277263572	5.86	24.89
ASI	WD	1	25	S1_302248506	S1_302412690	3	210	rs131368069	rs132076316	5.00	20.18
CL	WW	7	390	S7_139527836	S7_153755552	10	175	rs132618944	rs128354246	5.11	28.69
CL	WW	3	50	S3_221734528	rs129311303	10	0	S10_116919080	rs838325189	5.26	16.07
CL	WD	3	195	rs131368069	rs132076316	8	160	S8_173792353	S8_163276039	5.77	31.78
CW	WD	2	60	rs129243511	S2_229825946	8	220	S8_145945904	S8_148216407	5.13	37.43
TB	WD	5	270	rs129997839	rs130004128	5	295	S5_24426031	S5_29004727	5.10	22.50
TB	WD	2	55	rs129234757	rs129243510	5	255	S5_9853336	S5_10571036	5.19	18.89

RWC: Relative Water Content; QY: Quantum yield; AN: Net CO_2 assimilation rate; g_s: Stomatal conductance to water vapor; TR: Transpiration rate; LT: Leaf temperature; ASI: Anthesis-silking interval; CL: Cob length; CW: Cob Weight; TB: Total Biomass; WW: Well-watered; WD: Water-deficit stress; LOD: Logarithm of

the odds ratio, Threshold value was ≥ 5; PVE (%): Total phenotypic variance in percentage explained by the QTL; Add by Add[a] : Additive by additive effect - Negative additive effect value indicates the direction of favorable allele from donor parent

Table 4. QTL × E interaction in RIL population over two seasons (*rainy and post rainy*).

Trait	chromosome	Position	Left Marker	Right Marker	LOD (AbyE)	PVE (AbyE)	Add	AbyE_01	AbyE_02
AN	3	314	S3_169283017	S3_173528165	1.10	3.55	-1.09	0.84	-0.84
AN	3	324	S3_174361072	rs129386882	1.35	7.92	-1.22	-1.24	1.24
g_s	3	168	S3_5950551	S3_5721251	2.17	2.64	-0.01	0.01	-0.01
g_s	7	162	S7_166501967	rs130671858	0.00	3.28	0.02	0.01	-0.01
g_s	7	372	S7_139259301	S7_139259336	2.14	3.06	0.01	-0.01	0.01
g_s	8	232	S8_152991912	S8_123369436	2.65	5.46	0.00	-0.01	0.01
NDVI	2	174	rs812099243	rs822182360	0.06	2.61	-0.02	0.01	-0.01
NDVI	2	389	rs131350195	S2_66658066	0.03	3.07	0.02	-0.01	0.01
NKPR	2	559	S2_27769950	rs131322326	2.97	5.72	0.80	0.66	-0.66
NKPR	10	405	S10_137217074	S10_140006671	3.29	7.03	-0.66	-0.73	0.73
QY	1	94	rs131918389	S1_3764785	1.93	3.21	-0.01	-0.01	0.01
QY	3	597	S3_25909601	rs131365216	2.47	5.94	0.01	0.01	-0.01
TR	7	162	S7_166501967	rs130671858	1.48	7.34	0.45	0.32	-0.32

AN: Net CO_2 assimilation rate; g_s: Stomatal conductance to water vapor; NDVI: Normalized Difference Vegetation Index; NKPR: Number of kernels per row; QY: Quantum yield; TR: Transpiration rate; LOD (AbyE)-LOD score for additive and dominance by environment effects; PVE (AbyE)-Phenotypic variation explained by additive and dominance by environment effect at the current scanning position, AbyE_01-Additive and dominance by environment 1 effect at the current scanning position; AbyE_02-Additive and dominance by environment 2 effect at the current scanning position

Table 5. List of annotated genes present within the major QTL intervals of various morpho-physiological and yield traits

QTL name	Chr	Position (start - end) bp	Position of SNP	SNP	Gene Size (bp)	Locus ID	Annotation	Biological process / Molecular function
qRWC9-1	9	136739367 – 136742084	136739244	A	2403	*Zm00001eb395070*	WEB family protein	Chloroplast avoidance movement
		137977817 – 138007957	137996982	T	30089	*Zm00001eb395310*	Guanine nucleotide exchange factor SPIKE 1	Small GTPase mediated signal transduction
qCTD7-1	7	169113995-169118206	169120933	T	1211	*Zm00001eb325260*	AP2-EREBP-transcription factor 201	DNA-binding transcription factor activity
		169117963-169131418	169120933	T	10455	*Zm00001eb325270*	ARM repeat superfamily protein	Protein import into nucleus
qAN3-2	3	177560743-177565229	177563598	T	4112	*Zm00001eb146040*	Chloride channel protein	Chloride transmembrane transporter activity
qg$_s$6-1	6	126751036-126754755	126753475	A	3719	*Zm00001eb280280*	Brassinosteroid insensitive 1-associated receptor kinase 1	Protein serine/threonine kinase activity
		120726441-120737326	120736548	G	10885	*Zm00001eb278890*	Transcription elongation factor SPT5	mRNA binding/ transcription regulation
qTR7-1	7	166501156-166503762	166501967	G	2606	*Zm00001eb324180*	Sugar carrier protein C	Symporter activity
		166624336-166625597	166627700	T	1261	*Zm00001eb324240*	Chlorophyll a-b binding protein, chloroplastic	Photosynthesis, light harvesting in photosystem I
qPH8-1	8	155140339-155152583	155152093	A	12244	*Zm00001eb360480*	Glutamate synthase 1 [NADH] chloroplastic	L-glutamate biosynthesis via GLT pathway
		150379460-150387067	150387963	G	3331	*Zm00001eb359190*	Carbonic anhydrase	Carbon utilization
qCH1-2	1	6360197-6362989	6365045	A	2792	*Zm00001eb002270*	Glyceraldehyde phosphate dehydrogenase B1	Glucose metabolic process
qCL2-1	2	4538665-4548639	4548124	G	6974	*Zm00001eb067750*	Mha2 - plasma-membrane H^+ATPase2	Proton export across plasma membrane
		2223206-2224013	2226727	A	807	*Zm00001eb066440*	Gibberellin-regulated protein 2	NIL
qNKPR2-2	2	27760910-27766381	27769950	A	5471	*Zm00001eb076550*	1-phosphatidylinositol-3-phosphate 5-kinase	Phosphatidylinositol phosphate biosynthetic process
		27765547-27768722	27769950	A	3175	*Zm00001eb076560*	GDP-mannose transporter (GONST5)	Antiporter activity
		26017200-26019281	26018549	T	2081	*Zm00001eb076110*	WAT1-related protein	Transmembrane transporter activity
qNKPR10-1	10	137214761-137217464	137217074	A	2703	*Zm00001eb427960*	Putative WAK-related receptor-like protein kinase family protein	Cell surface receptor signaling pathway
		140005504-140009372	140006671	A	3868	*Zm00001eb428890*	G2-like-transcription factor 41,	DNA-binding transcription factor activity
qCW2-1	2	241494911-241497812	241498576	G	2651	*Zm00001eb117750*	Proline-rich receptor-like protein kinase PERK4	Protein serine/threonine kinase activity

RWC: Relative water content; CTD: Canopy temperature depression; AN: Net CO_2 assimilation rate; g_s: Stomatal conductance to water vapor; TR: Transpiration rate; PH: Plant height; CH: Cob height; CL: Cob length; NKPR: Number of kernels per row; CW: Cob weight; chr: Chromosome

Figures

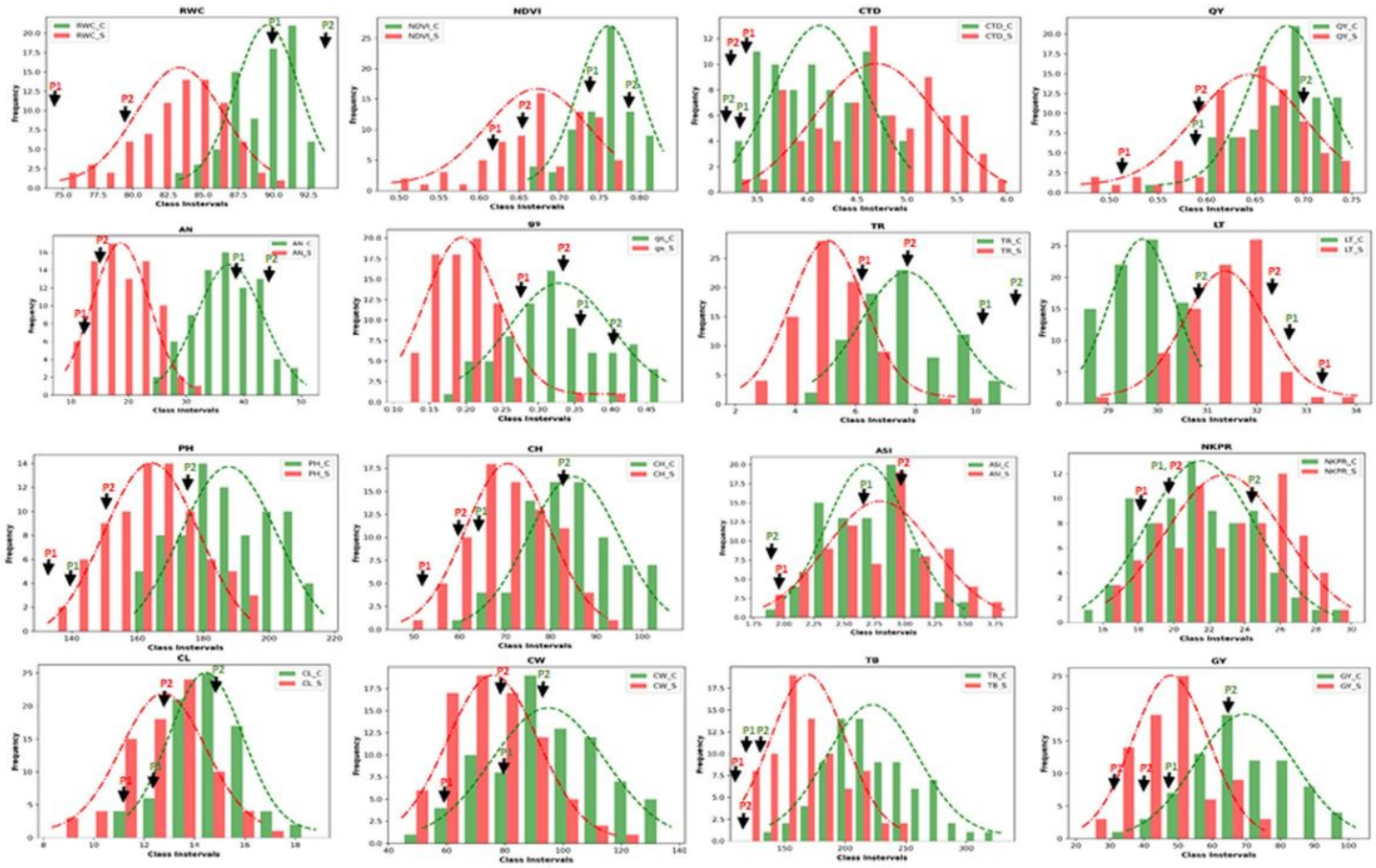

Figure 1

Frequency distribution of the phenotypic data of the RILs population of the various morpho-physiological and yield related traits. The values of the parents (P1 – HKI161 and P2 SNJ201126 are indicated by arrows, Well-watered: Green; Water-deficit stress: Red). RWC: Relative water content; NDVI: Normalized difference vegetation index; CTD: Canopy temperature depression; QY: Quantum yield; AN: Net CO_2 assimilation rate; g_s: Stomatal conductance to water vapor; TR: Transpiration rate; LT: Leaf temperature; PH: Plant height; CH: Cob height; ASI: Anthesis-silking interval; NKPR: Number of kernels per row; CL: Cob length; CW: Cob weight; TB: Total biomass; GY: Grain yield/ plant; C: Well-watered; S: Water-deficit stress

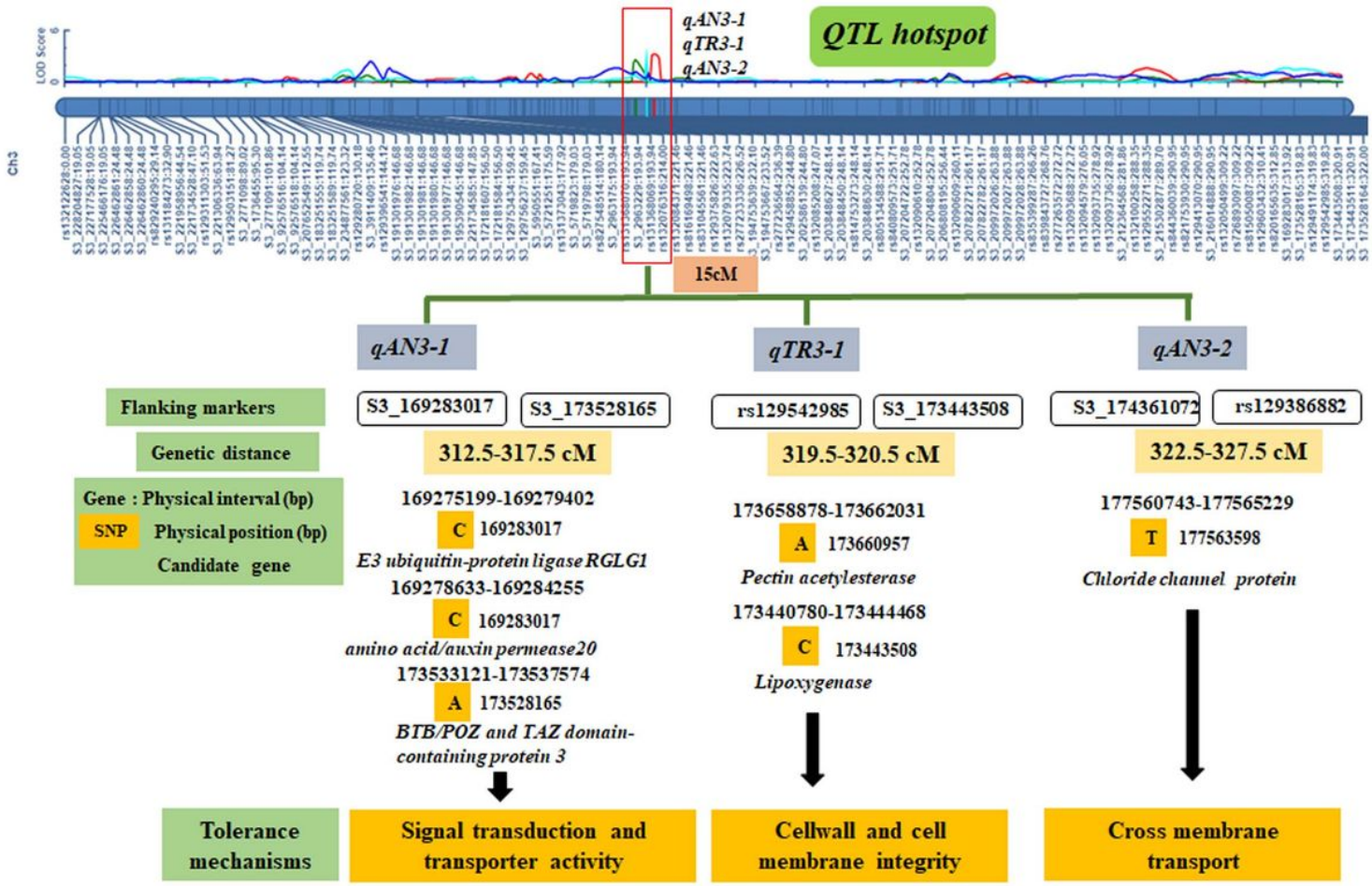

Figure 2

QTL hotspot identified for traits net CO_2 assimilation rate (AN) and transpiration rate (TR) using combined phenotyping data under well-watered and water-deficit stress conditions.

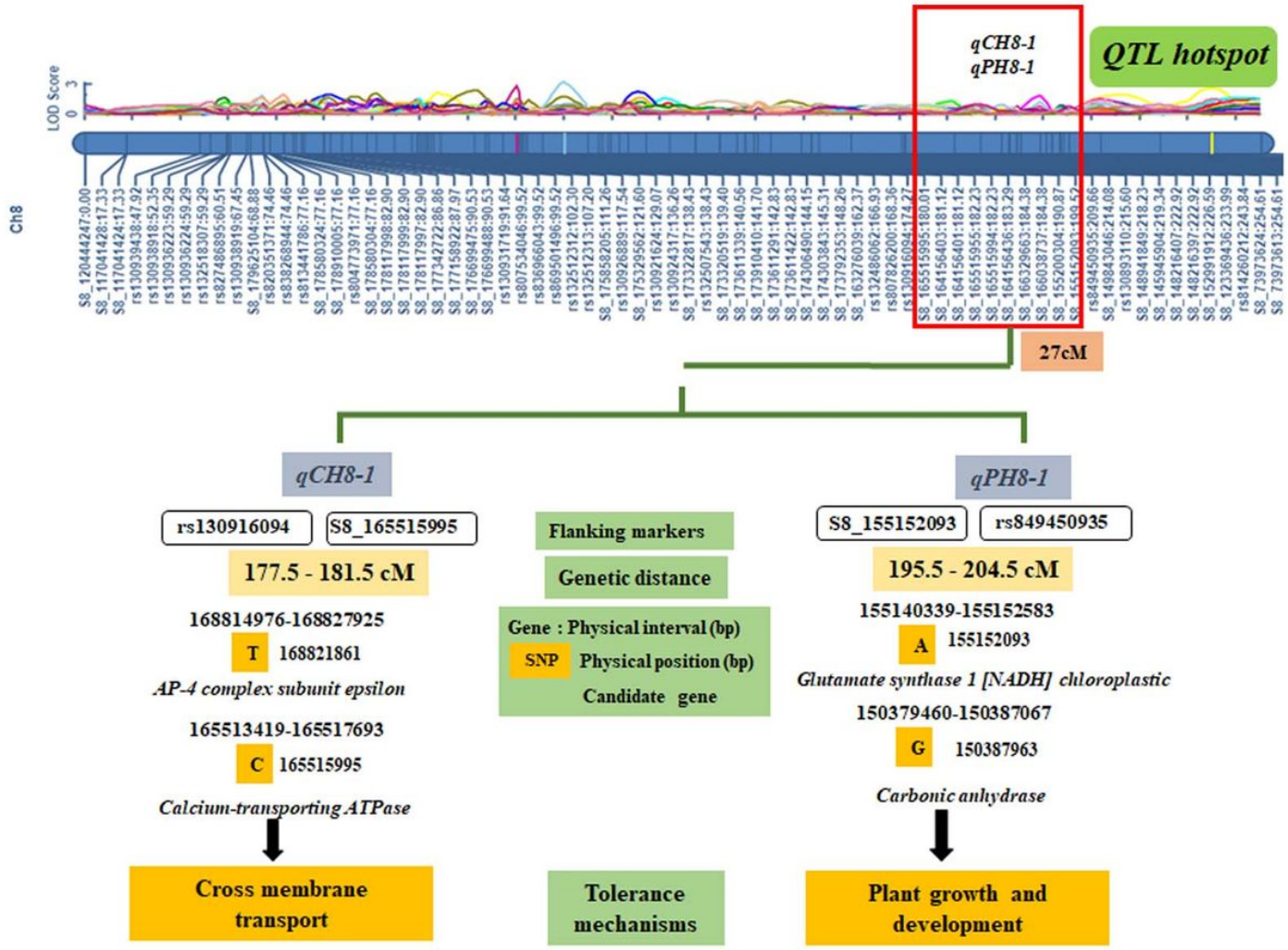

Figure 3

QTL hotspot identified for traits cob height (CH) and plant height (PH) using combined phenotyping data under well-watered and water-deficit stress conditions

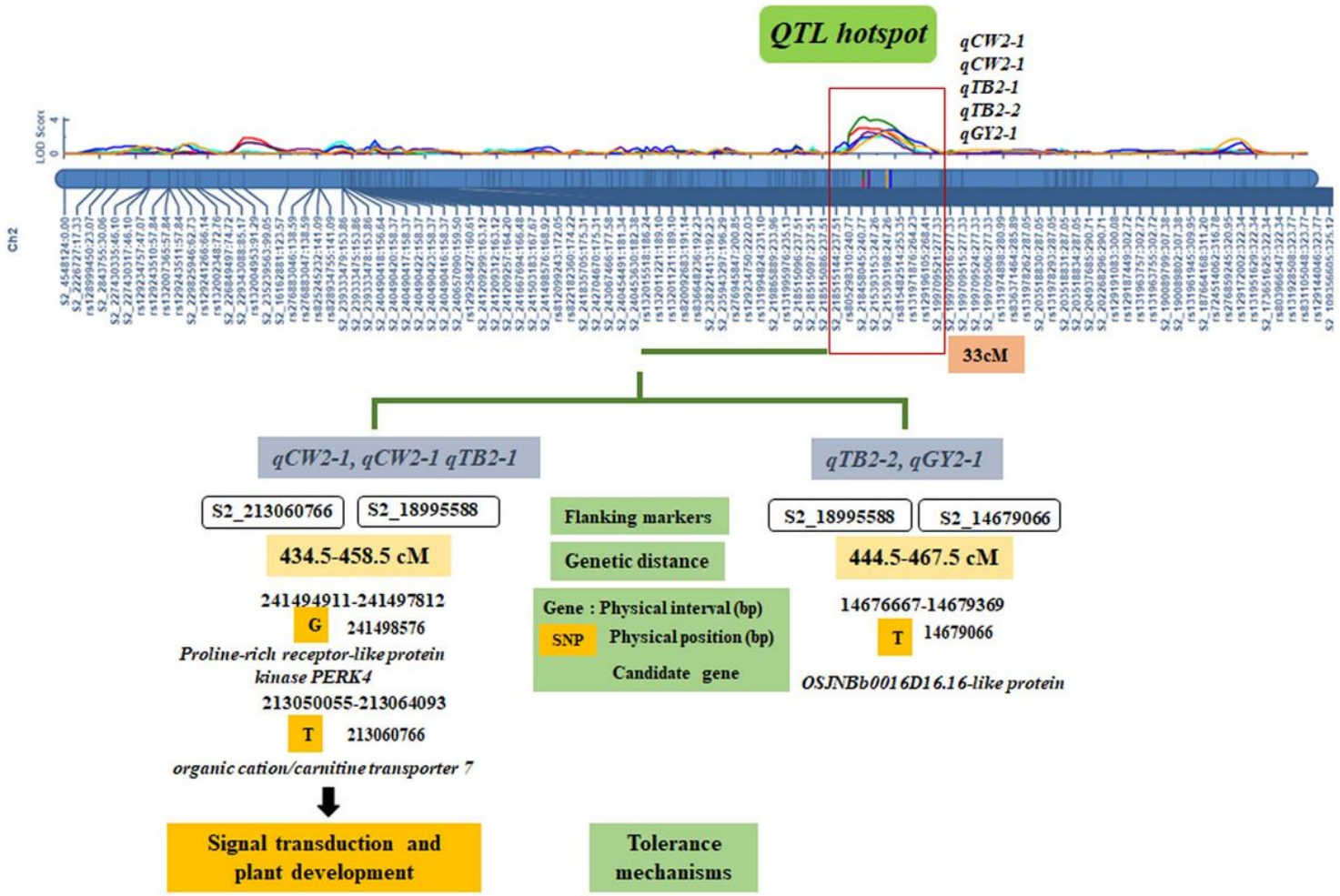

Figure 4

QTLs hotspot identified for cob weight (CW), total biomass (TB) and grain yield (GY) using combined phenotyping data under well-watered and water-deficit stress conditions

Supplementary Files

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