Mapping QTL For Seedling Morphological and Physiological Traits Under Normal and Salt Treatments in a RIL Wheat Population

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Abstract

Soil salinity seriously constrains wheat (*Triticum aestivum* L.) production globally by influencing its growth and development. To explore the genetic base of salt tolerance in wheat, a recombinant inbred line (RIL) population derived from a cross between high-yield wheat cultivar Zhongmai 175 (ZM175) and salt tolerant cultivar Xiaoyan 60 (XY60) was used to map QTL for seedling traits under normal and salt treatments based on a high-density genetic linkage map. A total of 158 stable additive QTL for 27 morphological and physiological traits were identified and distributed on all wheat chromosomes except 3A and 4D. They explained 2.35%–46.43% of the phenotypic variation with a LOD score range of 2.61–40.38. Among them, 39 QTL were detected under both normal and salt treatments, while 80 and another 39 QTL were detected under only normal and salt treatment, respectively. The alleles from XY60 increased corresponding traits for 100 QTL, while the alleles from ZM175 had positive effects for the other 58 QTL. Nearly half of the QTL (78/158) were mapped in nine QTL clusters on chromosomes 2A, 2B, 2D, 4B, 5A, 5B, 5D, and 7D (2), respectively. To prove the reliability and potentiality in molecular marker-assisted selection (MAS), seven QTL intervals were validated in two other genetic populations. In addition, besides additive QTL (*a*), epistatic (*aa*) and QTL-by-environment (*at*) interaction effects were also analyzed here. It was shown that 94 pairs of loci were detected with significant epistatic effect and 20 QTL were found to interact with treatment. This study provides a full elucidation of the genetic base of seedling traits (especially root system related traits) associated with salt tolerance in wheat, and the developed kompetitive allele specific PCR markers closely linked to stable QTL would supply strong supports to MAS in salt tolerant wheat breeding.

Key Message

The genetic base of 27 seedling traits under normal and salt treatments was fully analyzed in a RIL wheat population, and seven QTL intervals were validated in two other genetic populations.

Introduction

Soil salinity is one of the major abiotic stresses that decreases grain production and threatens food security worldwide (Flowers et al. 1997; Munns and Gilliham 2015; Roy et al. 2014). It was estimated that the global salt-affected land area was more than 800 million hectares (equal to 6% of the world's total land area) and it has been continuously increasing year by year due to climate changing, land clearing, and non-sustainable irrigation (Deinlein et al. 2014; Flowers and Yeo 1995; Munns and Tester 2008; Rengasamy 2010; Roy et al. 2014; Tester and Davenport 2003). Bread wheat (*Triticum aestivum* L.) is a moderately salt tolerant crops (Munns and Tester 2008) and grows most widely throughout the world (Matthew et al. 2011). To meet the food requirements, wheat production must increase by nearly 70% by 2050 (Foley et al. 2011; Tilman et al. 2002), however, it was estimated that the area of salinized arable land would also exceed 50% by then (Jamil et al. 2011).

Soil salinity damages plant growth severely by lowering growth rates, reducing tillers, accelerating the senescence of old leaves, decreasing photosynthesis capability, and affecting reproductive development, which would lead to significant reduction in final agricultural yield (Munns and Tester 2008; Roy et al. 2014). To resist salt stress, plants have evolved many mechanisms of salinity tolerance, which fall into three categories: osmotic tolerance (the ability of maintaining turgor by accumulating small molecular substances like organic acids, inorganic ions, carbohydrates, and amino acids), Na^+ exclusion (the ability of reducing net Na^+ from root to shoot), and tissue tolerance (the ability of maintaining tissue function after Na^+ and Cl^- concentrations elevated) (Munns and Tester 2008). Among them, Na^+ exclusion is the most intensively studied mainly because it is relatively straightforward to phenotype (Roy et al. 2014) and coincides with people's common perception. The *high affinity potassium* transporter (*HKT*) gene family (Ali et al. 2012; Byrt et al. 2007; Davenport et al. 2007; Hauser and Horie 2010; Horie et al. 2009; Huang et al. 2006; Munns and Tester 2008; Platten et al. 2006) and the salt overly sensitive (SOS) signaling pathway (Ji et al. 2013; Mahajan et al. 2008; Qiu et al. 2002; Shi et al. 2003; Weinl and Kudla 2009; Yang et al. 2009) played significant roles in regulating Na^+ transport. *Kna1* was the first major locus for salt tolerance in wheat, which controlled leaf Na^+ content and maintained a high K^+/Na^+ discrimination in leaf blades (Dubcovsky et al. 1996; Gorham et al. 1997; Gorham et al. 1987; Gorham et al. 1990; Luo et al. 1996). Further studies found that *HKT1;5-D* retrieving Na^+ from the xylem vessels in roots was the candidate gene of *Kna1* (Byrt et al. 2007; Byrt et al. 2014; Davenport et al. 2005). *Nax1* and *Nax2*, which were mapped to chromosomes 2AL and 5AL in durum wheat, respectively, contributed to low Na^+ concentration in leaf blades and also belonged to *HKT* family (Byrt et al. 2007; Huang et al. 2006; James et al. 2006; Lindsay et al. 2004; Munns et al. 2012). Various QTL for Na^+ content have been mapped in bread wheat in different studies (Asif et al. 2018; Devi et al. 2019; Genc et al. 2010; Genc et al. 2019; Xu et al. 2013), but only a few of them were co-localized with *Kna1*, *Nax1* (Hussain et al. 2017; Oyiga et al. 2018) or *Nax2* (Xu et al. 2013), even using the genome-wide association method (Genc et al. 2019). Therefore, the benefits of these QTL (genes) in bread wheat breeding were uncertain though *Nax2* could increase grain yield by 25% in durum wheat (Munns et al. 2012). Considering that the ultimate aim of salt tolerance breeding is to increase crops' ability to maintain growth and productivity in saline soils relative to that in non-saline soils (Roy et al. 2014), breeders usually concentrate on morphological and biomass related traits besides ions (Na^+, K^+, and Cl^-) content in mapping studies of wheat.

As the major organ for water and mineral nutrient absorption, root is the first tissue sensing osmotic stress and ion toxicity. Although root growth is usually less affected than leaf growth, the initiation of new seminal or lateral roots probably reduces with time (Munns and Tester 2008). Compared with the above-ground traits, little is known about the "hidden" root especially under salt stress in bread wheat. Recently, Fan et al. (2018) mapped QTL for root system architecture-related traits (RSATs) under high and low nitrogen environments, and found some chromosome regions responding to nitrogen deficiency and an interval on chromosome 7B controlling RSATs and thousand kernel weight concurrently. Soriano & Alvaro (2019) found that 35 meta-QTL were related to root architecture and/or drought stress response by meta-analysis with many published articles. For salt-tolerance studies in wheat, researchers formerly

focused on the QTL for the maximum root length and biomass (Devi et al. 2019; Ma et al. 2007; Xu et al. 2012; Xu et al. 2013), but few noticed the variation of RSATs under salt stress such as root diameter, main and lateral root number, length, surface area and so on.

Breeding improved varieties adapting to saline soil through molecular marker-assisted selection (MAS) has been lagging behind in bread wheat because of the complex mechanisms of salt tolerance and large genome sequence (~17 Gb). Although many loci for morphological and physiological traits were detected through QTL mapping (Asif et al. 2018; De Leon et al. 2011; Devi et al. 2019; Genc et al. 2013; Genc et al. 2010; Genc et al. 2019; Ghaedrahmati et al. 2014; Jahani et al. 2019; Ma et al. 2007; Masoudi et al. 2015; Nezhad et al. 2019; Quarrie et al. 2005; Xu et al. 2012; Xu et al. 2013), only a few of them were reported to have effects on final grain yield in bread wheat (Asif et al. 2018; Devi et al. 2019; Genc et al. 2013; Genc et al. 2019; Nezhad et al. 2019). There is still a huge gap between understanding the genetic basis of salinity tolerance in wheat and applying the available knowledge to deliver salt-resilient varieties subsequently (Mujeeb-Kazi et al. 2019). QTL mapping results for salt tolerance would be diverse in different populations, and even in the same population under various environments. Genetic background (GB) affects not only the detection but also the expression of QTL (Han et al. 2012; Jahani et al. 2019; Venuprasad et al. 2012; Vikram et al. 2011), thus GB hinders the universal utilization of QTL found in different backgrounds (Jahani et al. 2019). As important genetic components, epistatic effect and QTL × environment interaction effect affect most quantitative traits greatly (Xu and Crouch 2008). In order to comprehensively elucidate the identified QTL and successfully apply them to breeding program, researchers have been gradually aware of the importance of the epistasis and QTL-by-environment interaction in QTL mapping for salt tolerance in wheat (Jahani et al. 2019; Nezhad et al. 2019; Xu et al. 2012; Xu et al. 2013).

In this study, a recombinant inbred line (RIL) population derived from a cross Zhongmai 175 (ZM175) / Xiaoyan 60 (XY60) was used to map QTL for seedling traits of shoot and root under normal and salt treatments based on a high-density genetic linkage map constructed with a Wheat55K SNP array. Besides additive QTL (*a*), epistasis (*aa*) and QTL-by-environment (*at*) interaction effects were also analyzed. In addition, to identify true and stable QTL, we used the simple mean and best linear unbiased estimates (BLUE) data from three trials as phenotype performances and validated some QTL in two other genetic populations.

Materials And Methods

Plant materials

XY60 is a new derived cultivar of Xiaoyan 6 and has a steady drought and salt resistance. As a classic case of distant hybridization between wheat and *Thinopyrum ponticum* (2n = 10x = 70), Xiaoyan 6 was characterized with wide adaptability to multiple environments, high yield potential and excellent bread-making quality (Li et al. 2008). ZM175 is a main high-yield cultivar grown in the Northern Winter Wheat Region and Huanghuai Wheat Region of China with high water and nutrient use efficiency. A total of 254

lines from a recombinant inbred line (RIL) population derived from a cross between ZM175 and XY60 were used in the present study. In addition, a RIL population containing 182 lines derived from a hybrid between Xiaoyan 54 and Jing 411 (Xu et al. 2012) and a double haploid (DH) line population consisting of 150 lines derived from a cross between Hanxuan 10 and Lumai 14 (Hao et al. 2003) were involved in this study as well. It is worth noting that Xiaoyan 54 is a parent of XY60 and Jing 411 is a parent of ZM175.

Methods

The experiment was carried out in the greenhouse at Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. The salt tolerance of 254 RILs and their parents was evaluated in hydroponic culture at two salt concentrations (0 and 150 mM NaCl, designated as the normal (CK) and salt stress (S) treatment, respectively) and three trials (CK1, S1, CK2, S2, CK3 and S3) was conducted. Fifteen plump seeds of each line were surface-sterilized in 10% H_2O_2 for 30 min, rinsed with deionized water and then germinated on grid net for 7 days. The eight most uniform seedlings of each line were selected and departed into CK and S groups averagely. Then they were transplanted into opaque plastic boxes and attached to the cover of the boxes using soft sponge rubber after the residual endosperm being removed. One box contained 15 L nutrition solution (Table S1) with 24 holes (4 seedlings per hole) evenly distributed on its cover. From the next day, 50 mM NaCl was added into the solution every day until to the final concentration of 150 mM for S treatment. The solution was renewed every 3 days with pH = 5.8–6.0. Plastic boxes were randomly placed and rearranged when the solution was renewed. The greenhouse maintained under a 16/8-h light/darkness cycle at 22°C/18°C during the plant growing. About three weeks later, all the plants were harvested after measuring the chlorophyll content of the first leaf.

Traits measurement

The chlorophyll content of each plant was measured using a leaf chlorophyll meter (Soil and Plant Analyzer Development, SPAD-502, Minolta, Osaka, Japan). For each plant, the SPAD was derived from the average of three readings at the base, middle and tip of the first leaf. Tiller number (TN), leaf number (LN), and yellow leaf number (YN) were counted. Shoot height (SH) and maximum root length (RL) were measured with a ruler. Fresh weight of shoot (SFW), dry weight of shoot (SDW), dry weight of root (RDW) and total dry weight (TDW) were measured with an electronic balance. Root system architecture-related traits (RSATs) were analyzed using a WinRHIZO software developed by Regent Instruments Canada Inc. (Ottawa, ON, Canada). The root morphological parameters included total root tip number (TRT), total root average diameter (TRAD), total root length (TRL), total root surface area (TRSA), main root tip number (MRT), main root length (MRL), main root surface area (MRSA), lateral root tip number (LRT), lateral root length (LRL) and lateral root surface area (LRSA). Main root means its average diameter was > 0.300 mm and <= 0.850 mm and lateral root means its average diameter was > 0.060 mm and <= 0.300 mm. The detection method of K^+ and Na^+ concentration was as follows: mixed and triturated sample (25-30 mg) from four dry plants of each line was dissolved in a nitric acid solution (13 mL HNO_3 and 2 mL H_2O_2)

using an advanced microwave digestion system (ETHOS 1, Milestone S.r.l., Shelton, CT, USA). After that, the concentration of K^+ and Na^+ in shoot (sK and sNa) and root (rK and rNa, only in the first trial) were assayed using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES, Optima 5300DV, PerkinElmer, Waltham, Massachusetts, USA). K^+/Na^+ ratio in shoot (sK/Na) and root (rK/Na) were calculated based on the concentration of K^+ and Na^+.

Statistical and QTL mapping

Correlation analysis was performed by SPSS Statistics software (IBM SPSS Statistics 23.0, Chicago, IL, United States). Analysis of variance (ANOVA) and narrow-sense heritability (h^2) for all traits under CK and S treatments were analyzed in the IciMapping 4.1 (http://www.isbreeding.net/software/?type=detail&id=18) with the ANOVA function. A total of ten phenotype data sets, which contained the average of each trial (CK1, CK2, CK3, S1, S2, and S3) as well as the simple mean (CKMean and SMean) and BLUE (CKBlue and SBlue) of three trials, were used to map QTL. The high-density genetic linkage map of the "ZM175 / XY60" RIL population was constructed utilizing a Wheat55K SNP array, which spanning 3250.71 cM included 2437 bin markers from 16008 SNPs distributed on 21 chromosomes (Luo et al. 2021). The chromosome length ranged from 85.99 cM (chromosome 4B) to 198.45 cM (chromosome 5D), and the average length was 154.80 cM. The density of bin markers was 1.33 cM with 116 bins on each chromosome averagely. Two softwares IciMapping 4.1 and WinQTLCart 2.5 (https://brcwebportal.cos.ncsu.edu/qtlcart/WQTLCart.htm) were used to map QTL with different methods. Pre-adjusted mapping parameters for Icimapping 4.1 were set: method = inclusive composite interval mapping (ICIM), step = 1.0 cM, PIN = 0.001, and logarithm of the odds (LOD) $\geq$ 2.5. For WinQTLCart 2.5, parameters setting were as follows: method = composite interval mapping (CIM), walk speed = 0.1 cM and threshold = 11.5. The epistatic effect *aa* and the interaction effect between QTL and treatment (*at*) were also analyzed with IciMapping 4.1. Additive QTL was named as "*Q*" plus trait name along with the chromosome information at the end, and "*c*" was added in the front of QTL for those with environment interaction effect.

Conversion of SNPs to kompetitive allele specific PCR (KASP) markers

Based on the sequences, key SNPs linked to major QTL were successfully converted into KASP markers, the specific technology for SNP genotyping. According to the manufacturer's instructions, the designed KASP markers were evaluated for their polymorphisms. KASP reactions were performed in a StepOnePlus Real-time PCR System (Applied Biosystems, USA), and fluorescence was analyzed using corresponding StepOne Software v2.3.

Result

Phenotypic variation, performance and correlation

Phenotypic characters (27 traits) of the "ZM175 / XY60" (ZX) RIL population and their parents were investigated under CK and S treatments in three trials. Based on the correlation analysis, significant

positive relationships were found among three trials (Table S2). For most traits, correlation coefficients were about 0.4–0.7 under CK treatments and 0.3–0.6 under S treatments. The coefficients for the ionic traits (K^+, Na^+ and K^+/Na^+ ratio) were less than 0.4 indicating their vulnerability to environmental influences. The phenotypes of 254 RILs and their parents and narrow-sense heritability (h^2) of all traits were summarized in Table 1. According to our experiments, the parental lines ZM175 and XY60 were significantly different in root related traits (RL, TRL, TRSA, TRT, TRAD, LRL, LRSA, LRT, MRL and MRT), SPAD and the cation contents. Although all root related traits were significantly inhibited under S treatments, XY60 had a more developed root system than ZM175 regardless of salt levels. After suffering salt stress, XY60's old leaves still stayed green while those of ZM175 became yellow or even died. The K^+ concentration and K^+/Na^+ ratio in XY60's shoot were higher than those in ZM175, whereas the opposite result occurred for Na^+ concentration. Under salt stress, the maximum, minimum and average values of the RILs for all seedling traits except YN and Na^+ concentration decreased distinctly compared with those under CK treatment. The h^2 for all measured traits were also obviously declined when the plants were treated with salt stress. For most traits, the skewness and kurtosis were small (less than 1.0) which demonstrated that the phenotype values followed normal distribution. In conclusion, ANOVA indicated that treatments, genotypes and genotype × treatment interaction significantly affected all of the traits related to seedling growth.

Correlation analysis was also carried out among different traits (Table S3). SFW, SDW and RDW presented significant and positive correlations with TDW and the correlation coefficients were more than 0.8 under CK and S treatments. SH, TN, LN, SPAD and RSATs were also positively correlated with TDW, while YN and sNa were negatively correlated with TDW. It was reasonable that there existed a positive correlation between sK and TDW under S treatment, but it was an exception that they were negatively related under CK treatment. The correlation between SH, RL, SPAD, TRT, LRT, RDW and TDW became higher under S treatment compared with those under CK treatment. In addition, obviously positive correlations were observed between sNa and YN under S treatment and between sK and SWC under CK treatment.

QTL mapping

In this study, two mapping softwares (IciMapping and WinQTLCart) were firstly used to detect the QTL for 27 seedling traits with the simple mean and BLUE values of three trials. It was shown that about 70% of the QTL detected by two softwares were the same, and the major QTL were hardly different (not shown). Then, QTL for all traits were detected by IciMapping with data sets of each trial (CK1, CK2, CK3, S1, S2, and S3). It was found that 153 repeatable QTL (detected with two or more data sets) and 5 QTL for rK, rNa and rK/Na (rK and rNa were assayed only in the first trial) were distributed on all wheat chromosomes but 3A and 4D (Table 2). These loci individually explained 2.35%–46.43% of the total phenotypic variation with LOD scores ranging from 2.61 to 40.38. Among them, 39 QTL were detected under both CK and S treatments, while 80 and another 39 QTL were detected under only CK and S treatment, respectively. A total of 12 QTL could explain more than 10% of the phenotypic variation and 80 QTL

explained 5%–10% of the phenotypic variation. The additive effects of 100 QTL were derived from XY60 alleles, whereas the effects of the other 58 QTL were from ZM175 alleles. Epistatic effect analysis showed that a total of 94 pairs of loci mainly for YN, SPAD, TRAD, and MRT were detected but none was co-localized with the additive QTL. In particular, most of them just explained little phenotypic variation, and only 10 pairs of loci explained more than 2% of the phenotypic variation (Table S4). Here, a total of 20 QTL were found to interact with treatment (Table 3), and 19 of them were major additive QTL in Table 2. Among them, the interaction effects at five loci explained over 10% of the phenotypic variation, especially the interactions between *cQRl-2B* and treatment (25.98%) and between *cQSh-4B* and treatment (20.11%).

Seven and nine QTL were detected for SH and RL respectively. Among them, *QSh-4B.2* and *QRl-2B.1* were detected with significant additive × treatment (*at*) effects. *QSh-4B.2* explained the maximum phenotypic variation (36.55%) with a LOD score of 29.35. However, it was only detected under CK treatment. Interestingly, *QSh-4B.1* was found under S treatment nearby *QSh-4B.2*. Similarly, *QRl-2B.1* could explain the maximum phenotypic variation (46.43%) with a LOD score of 38.4 under CK treatment; while *QRl-2B.2* was detected 14 cM away from *QRl-2B.1* under both CK and S treatments. Eight and 12 QTL were detected for TN and LN, respectively. Three QTL for TN (*QTn-2A*, *QTn-2D*, *QTn-5B)* and six for LN (*QLn-2A.2*, *QLn-2D.2*, *QLn-3D*, *QLn-5A*, *QLn-5B.1* and *QLn-6A*) were detected under both CK and S treatments, and *QTn-7A*, *QTn-7B*, *QLn-2D.1*, *QLn-5B.2* and *QLn-6D-1* were discovered only under S treatments. *QTn-5B* and *QLn-2B* explained the maximum phenotypic variation for corresponding traits. A total of six QTL (*QTn-5B*, *QLn-2A.2*, *Ln-2D.1*, *QLn-5A*, *QLn-5B.1* and *QLn-6A*) were found with significant *at* effects, but they only explained a little phenotypic variation (<3%).

For shoot and root biomass related traits (SFW, SDW, RDW and TDW), there were seven, five, nine and six QTL detected, respectively. Among them, three intervals on chromosomes 2A, 2B and 5A were found to contribute to all the four biomass related traits, and two intervals on 1B and 4B were proved to be related with all biomass traits but RDW under both treatments or only under S treatment. For SWC, nine QTL were mapped on chromosomes 1B, 2B (2), 2D, 3B, 4B, 5B, 6B and 7B. *QSwc-4B* explained the maximum phenotypic variation with a LOD score of 6.94 under CK treatment. *QSwc-6B* was detected under both CK and S treatments and explained 7.20% of the phenotypic variation.

After salt treatment, Na^+ content in root and shoot tissues increased rapidly while K^+ absorbing capacity decreased. It was previously verified that K^+ and Na^+ concentrations and K^+/Na^+ discrimination were very important to salt tolerance (Byrt et al. 2014; Dubcovsky et al. 1996; Dvorak and Gorham 1992; Gorham et al. 1987; Lindsay et al. 2004; Munns et al. 2012; Shah et al. 1987). Here, cation (K^+ and Na^+) contents in root and shoot were assayed in one and three trials, respectively. We found that the QTL (on chromosomes 1D, 3B, 5D and 6A) for K^+, Na^+ and K^+/Na^+ ratio in root were completely different from those (on chromosomes 1D, 2B, 2D, 4B, 5D, 6A and 6B) in shoot. Although QTL for Na^+ in shoot were discovered with only one data set, two QTL (*QsNa-2B* and *QsNa-5A*) could be detected with the mean value by both mapping softwares (Fig. S1). Interestingly, *QsNa-2B*, *QsK-2B* and *QsK/Na-2B* were mapped

to the same interval of 0–3.5-cM on chromosome 2B under CK and S treatments or just S treatment. *QsK-4B* and *QsK/Na-4B* were mapped to the same interval (21.5–34.5 cM) on the chromosome 4B, and they could explain the maximum phenotypic variation. It has been shown that sNa was positively correlated with YN, while they had a significantly negative relationship with SPAD under S treatment (Table S3). Here, *QSpad-1A* and *QYn-1A* were detected in the same interval 37.5–40.5 cM on chromosome 1A and *QSpad-3D* and *QYn-3D.1* were co-located in 74.5–81.5 cM on chromosome 3D. Coincidently, the additive effects of *QSpad-1A* and *QSpad-3D* were derived from XY60 alleles, while the additive effects came from ZM175 alleles at *QYn-1A* and *QYn-3D.1*. In addition, *QSpad-1A* explained the maximum phenotypic variation under S treatment and *QYn-1A* had significant *at* effects.

For 10 RSATs, a total of 60 QTL were detected. Among these, 13 QTL were discovered under both CK and S treatments, 37 and 10 QTL were under only CK and S treatment, respectively. Besides, nine (*QTrt-5A*, *QTrt-5D*, *QTrad-2B*, *QTrsa-2A*, *QTrsa-2B*, *QMrl-2B*, *QLrl-5A*, *QLrt-5A* and *QLrt-5D*) of all 60 QTL had significant *at* effects. The *at* effects of *cQLrl-5A* and *cQLrsa-5A* explained more than 10% of the phenotypic variation. Significantly, two chromosome intervals (i.e., 0–4.5 cM on chromosome 2B and 29.5–40.5 cM on chromosome 2A) were significantly important for root related traits. The interval on chromosome 2B contributed to all the root related traits except MRT, and it could explain the maximum phenotypic variation for all the traits but TRT and LRT. The interval on chromosome 2A was related to all the root traits except TRAD and MRT, and it could stably explain 5%–10% of the phenotypic variation. In addition, chromosome 7B was important for root tip number. *QTrt-7B* and *QLrt-7B* were mapped in the same interval (67.5–69.5 cM) and *QMrt-7B* was close to them in 78.5–80.5 cM.

QTL clusters

QTL for different traits could clustered together in one interval on a certain chromosome, which was usually pleiotropic and important. In the present study, nearly half of the QTL (78/158) were identified to gather on group-2 and -5 chromosomes, as well as chromosomes 4B and 7D (Fig. 1), which were designated as C2A, C2B, C2D, C5A, C5B, C5D, C4B, C7D-1 and C7D-2, respectively. In C2A, there were 14 QTL for LN, RL, SFW, SDW, RDW, TDW and RSATs (TRL, TRT, TRSA, MRL, MRSA, LRL, LRT and LRSA) in the interval of 27.5–48.5 cM. The additive effects of them were all from XY60 alleles. Only two QTL had significant *at* effects, which could just explain 3.94% (*QTrsa-2A*) and 0.21% (*QLn-2A*) of the phenotypic variation. In C2B, a total of 19 QTL for TN, LN, SPAD, RL, sK, sK/Na, SFW, SDW, RDW, TDW and RSATs (TRL, TRT, TRAD, TRSA, MRL, MRSA, LRL, LRT and LRSA) were in the region of 0–4.5 cM, and their additive effects were all derived from XY60 alleles, too. The *at* effects of *cQRl-2B.1*, *cQTrsa-2B*, *cQTrad-2B* and *cQMrl-2B* explained 25.98%, 5.47%, 7.96% and 4.40% of the phenotypic variation, respectively. In the interval 117.5–141.5 cM, six QTL for SH, TN, LN, YN, sK and SWC assembled to form C2D. None of these six QTL had significant *at* effect, and the additive effects of them except for *QSh-2D* were derived from ZM175 alleles. Seven QTL for SH, LN, sK, sK/Na, SDW, SWC and TDW were located in C4B (17.5–33.5 cM). *QSh-4B.2* and *QsK-4B* had significant *at* effects (20.11% and 12.14%) as well as high additive effects (36.55% and 12.87%). There were 10 QTL for TN, LN, SFW, SDW, RDW, TDW and RSATs (TRL, TRSA, MRSA and LRSA) in the block of 18.5–39.5 cM on chromosome 5A (C5A), at which the ZM175-

derived alleles had positive effects on corresponding traits. Only *QLn-5A* was observed with significant *at* effect explaining 0.2% of the phenotypic variation. In C5B, four QTL for TN, LN, RDW, and MRSA clustered in the region of 39.5–55.5 cM, and the alleles from XY60 expressed positive effects on the corresponding traits. Among them, *QTn-5B* was detected under both CK and S treatments, and its additive effect could explain 8.80% of the phenotypic variation while its *at* effect only explained 2.81%. *QLn-5B.1* was also detected under both treatments, and it contributed 5.19% to the phenotypic variation with just 0.05% of the *at* effects. The positions of ten QTL (*QRdw-5D*, *QsK-5D*, *QTrl-5D*, *QTrt-5D*, *QTrsa-5D*, *QMrl-5D*, *QMrsa-5D*, *QLrl-5D*, *QLrt-5D* and *QLrsa-5D*) on chromosome 5D were not very consistent for different data sets which led to a wide physical distance (124.5–185.5 cM). But their additive effects were all from XY60 alleles. Two QTL clusters (C7D-1 and C7D-2) were found in 57.5–58.5 cM and 115.5–119.5 cM on chromosome 7D, respectively. All QTL in them were for RSATs, and they only explained 2–5% of the phenotypic variation with no significant *at* effects. The alleles from XY60 at all the four QTL (*QTrt-7D*, *QLrl-7D.1*, *QLrt-7D* and *QLrsa-7D*) in the cluster C7D-1 could increase the corresponding traits values, while the alleles from ZM175 at all the five QTL (*QRdw-7D*, *QTrsa-7D*, *QMrl-7D*, *QMrsa-7D* and *QLrl-7D.2*) in the cluster C7D-2 showed positive effects. Fortunately, the additive effects of QTL above in one cluster usually derived from a same parent's alleles, which would promote their effective utilization.

Validation of the QTL

Although most QTL were simultaneously detected by different mapping softwares, we valuated them in "Hanxuan 10 / Lumai 14" (LH) DH population and "Xiaoyan 54 / Jing 411" (XJ) RIL population. Here, the additive effects of seven QTL intervals were verified (Fig. 2, Fig. S2 and Table S5). QTL for SFW, SDW and TDW on chromosome 1B were detected in the same interval in LH population as well, explaining the phenotypic variation by 2.56%, 6.46% and 8.88%, respectively (Table S5). Moreover, two common SNP markers (AX-109819289 and AX-108785293) linked to these QTL were found in ZX and LH populations (Fig. 2). *QLn-6A* was found to be linked to five common SNPs in ZX and LH populations. On chromosome 2B, QTL for TDW was detected in 13.45–14.15 cM in LH population and QTL for SPAD and root traits were found in 70.5–77.5 cM in XJ population, which sharing many SNPs with those in ZX population. It was worth mentioning that *QRl-2B*(XJCK), *QTrl-2B*(XJCK) and *QTrsa-2B*(XJCK) explained extensive phenotypic variation (42.20%, 24.56% and 14.46%, respectively) in XJ population, which was similar with those in ZX population. QTL for SH, SDW, TDW and SWC on chromosome 4B were discovered to be linked to eight identical SNP markers between ZX and XJ populations. In particular, *QSh-4B*(XJCK) could explain remarkable phenotypic variation (31.74%) in XJ population as *QSh-4B.2* (36.55%) in ZX population. On chromosome 5B, QTL for TN was detected under S treatment in both ZX and XJ populations, and two common SNPs (AX-109928742 and AX-89400290) were linked to it. QTL for SWC detected under both CK and S treatments were found in ZX and XJ populations and linked to 12 same SNP markers (Fig. S2).

KASP markers developing

To apply important QTL associated with salt tolerance to wheat breeding, six SNPs, i.e., AX-109383322 (1A) linked to *QSpad-1A* and *QYn-1A*, AX-109819289 (1B) linked to QTL for biomass (*QSfw-1B*, *QSdw-1B* and *QTdw-1B*), AX-109366069 (2A) linked to QTL for RSATs (*QRl-2A*, *QTrl-2A*, *QTrsa-2A* and *QTrt-2A*), AX-111606522 (2B) linked to QTL for root related traits (*QRl-2B.1*, *QTrl-2B*, *QTrad-2B,QTrsa-2B* and *QTrt-2B*) , AX-110967528 (5B) linked to *QTn-5B* and AX-109593935 (6A) linked to *QLn-6A* were successfully converted to KASP markers (Fig. S4 and Table S6), which would also play a role in the process of gene cloning.

Discussion

Seedling is very sensitive to salt stress in wheat life cycle. In spring, when winter wheat seedlings at reviving stage grow rapidly to enter erecting period, they have to survive the high salinity of surface soil due to water evaporation and saline accumulation in monsoon climate region. Thus, as the beginning of breeding salt-tolerant wheat cultivar, screening plants with strong salt tolerance at seedling stage is very pivotal.

It is known that K^+, Na^+ concentration and their ratio are very important for the salinity tolerance. Na^+ inhibits K^+ uptake and competes its binding sites in enzymes due to their physicochemical similarity. As a major gene enhancing K^+/Na^+ ratio in wheat, *Kna1* was found to be located at the long arm terminal of chromosome 4D (Byrt et al. 2014; Davenport et al. 2007; Dubcovsky et al. 1996; Dvorak and Gorham 1992; Gorham et al. 1997; Gorham et al. 1987) and it could be a critical reason why hexaploid wheat is more tolerant to salinity than durum wheat (Colmer et al. 2006; Gorham et al. 1987). In this study, no QTL was detected on chromosome 4D, probably because there was no difference in *Kna1* between two parental lines. Furthermore, *Kna1* hasn't been reported in any hexaploid wheat populations previously (Do et al. 2018; Genc et al. 2010; Genc et al. 2019; Ilyas et al. 2020; Jahani et al. 2019; Nezhad et al. 2019; Xu et al. 2012; Xu et al. 2013), indicating that this gene could exist in ancient hexaploid bread wheat. Although K^+ and Na^+ concentrations in XY60 and ZM175 were significantly different, no QTL for Na^+ exclusion was co-localized with *Nax1* (Huang et al. 2006; James et al. 2006; Lindsay et al. 2004) and *Nax2* (Byrt et al. 2007; James et al. 2006; Munns et al. 2012) in our study. However, we found a cation transporter gene *TraesCS2D02G428300* annotated as *HKT7* in the QTL cluster C2D (529662115–613348655). Besides, *QsK-2B*, *QsNa-2B* and *QsK/Na-2B* were co-located with QTL for biomass related traits (TN, LN, SFW, SDW, RDW and TDW). Similarly, *QsK*-4B and *QsK/Na-4B* were also co-localized with QTL for biomass related traits (SH, LN, SDW and TDW), and *TraesCS4B01G043100.1* in the interval of *QSh-4B* was annotated as *Rht-B1* by UniProt. Significantly, this region also mapped multiple QTL in dry salinity field, which contained QTL for plant height (PH), spike number per plant (SN), spikelet number per spike (SPS), kernel number per spike (KPS), thousand kernel weight (TKW), grain number per plant (GN) and harvest index (HI) (Luo et al. 2021). In addition, *QrNa-6A* for Na^+ concentration in root tissue could be the same locus as *Q.Na6A* (*cfd080– barc171*) (Genc et al. 2010) based on their physical positions. Their smaller correlation coefficients among three trials and lower h^2 proved that K^+ and Na^+ contents in seedling shoot were easily affected by environments, which maybe one reason why major stable genes

like *Nax1* and *Nax2* were not detected in this study. The increased senescence rate of old leaves could be considered as Na^+-specific toxicity symptom due to either high leaf Na^+ or low tolerance to the accumulated Na^+ (Munns and Tester 2008). Consistently, YN was positively correlated with sNa, while SPAD of the first leave was negatively correlated with sNa, which was accordant with previous study (Masoudi et al. 2015). In ZX population, YN and SPAD were more stable than sK and sNa according to their higher correlation coefficients among three trials and narrow-sense heritability. Thus, YN and SPAD could be good indicators of sNa, and the regions 37.5–40.5 cM (*QYn-1A* and *QSpad-1A*) on chromosome 1A and 74.5–81.5 cM (*QYn-3D* and *QSpad-3D.1*) on chromosome 3D deserved further study. Specifically, gene prediction and functional annotation showed that *QSpad-1A* contained some genes like potassium transporter, H-ATPase 3, calcium-transporting ATPase and glutathione S-transferase according to IWGSC RefSeq v1.0 (Table S7).

Root plays an important role in seedling biomass under salt stress, but there is a lack of systemic study on the morphological characters of root after wheat suffering salt treatment. In this study, we noticed that root related traits (RL, TRL, MRL, LRL, TRSA, MRSA and LRSA) had higher correlation coefficients (more than 0.5) with TDW under S treatment, and RL, TRT, LRT and RDW even had higher correlation coefficients with TDW under S treatment than under CK treatment (Table S3). It was also demonstrated that QTL for root traits could be co-localized with QTL for biomass related traits. For example, QTL for RSATs and biomass traits were co-located at the short arm terminal of chromosome 2B, which consisted of genes like WRKY transcription factor, ethylene receptor, jasmonate-induced protein, defensin and so on (Table S7). Based on the physical positions of the linked markers, we determined that this region was previously reported (Cao et al. 2014; Ren et al. 2012) to benefit phosphate nutrient uptake and biomass accumulation and contribute to root length significantly but hinder root diameter under N deficiency. On chromosome 2D, QTL for root related traits (RDW, TRL, TRT, LRL, LRSA and LRT) were detected in 11.5–39.5 cM under S treatment in this study. Similarly, Xu et al. (2012) found QTL for root, shoot and total dry weight under both CK and S treatments in the interval *Xcfd53*–*Xwmc112* on chromosome 2D. Additionally, it was noticed that QTL for K^+, proline content, tiller number (TN), TKW, days to heading (DTH), and days to anthsis (DTA) were also detected under sodic stress in this region (Devi et al. 2019). Moreover, it was analyzed that the QTL region on chromosome 2D in the present study matched with those in the above two studies based on the physical positions of linked markers. Notably, compared with our previous study (Luo et al. 2021), QTL for “hidden” underground traits at seedling stage were mapped to the same chromosome regions with some QTL for observable aboveground traits at adult stage. Examples are as follows: *QTrt-5A* (57.5–74.5 cM), *QLrt-5A* (57.5–74.5 cM) and *QLrl-5A* (62.5–75.5 cM) were mapped to the similar interval on chromosome 5A as *QSps-5A* (54.5–61.5 cM) and *QGn-5A* (59.5–80.5 cM). Like QTL for RSATs and seedling biomass traits, *QHi-5A* (33.5–35.5 cM) in dry salinity field was also detected in the region of C5A (18.5–39.5cM). QTL for RSATs (*QTrt-5D*, *QTrl-5D*, *QTrsa-5D*, *QMrsa-5D*, *QLrt-5D* and *QLrl-5D*) on chromosome 5D were co-localized with *QSps-5D* (146.5–173.5 cM). *QRdw-6A* and *QMrsa-6A* were mapped to the same region as QTL for PH, spike length (SL), SPS, KPS, TKW and kernel related traits (kernel length, kernel width and perimeter of kernel). Besides, *QRl-7B*, *QTrt-7B*, *QTrad-7B* and *QLrt-7B* were also co-localized with QTL for kernel length. Fan et al. (2018) found that QTL for

RSATs were clustered in the 82.50–97.50-cM interval of chromosome 7B, which also had a significant effect on TKW. Based on our analysis, three chromosome regions respectively on chromosome 5A, 5D and 6A controlled both RSATs and SPS. Thus, root traits could not only improve the seedling biomass under salt treatment, but also contribute to yield related traits on saline soil. Accordingly, selecting plants with favorable alleles for seedling growth traits especially RSATs under salt treatment could be useful for the final grain yield in salinity field.

In this study, more than half (87/158) of the QTL were located on group-2 and − 5 chromosomes (Fig. S3). It has been reported that group-5 chromosomes were regarded to carry genes for abiotic stress resistance, including salt tolerance in wheat (Cattivelli et al. 2002; Quarrie et al. 2005). *Nax1* (*HKT7*) (Huang et al. 2006; Lindsay et al. 2004) and *Nax2* (*HKT8*) (Byrt et al. 2007; Munns et al. 2012) were located at the long arm terminals of chromosomes 2A and 5A, respectively. Interestingly, a homologous gene of *HKT7* was found in the cluster C2D in the present study. Significantly, the cluster C2D (117.5–141.5 cM) contained not only QTL at seedling stage like *QSh-2D*, *QTn-2D*, *QLn-2D*, *QSwc-2D* and *QsK-2D* under salt stress, but also QTL for SPS, TKW, yield per plant (YPP), aboveground biomass per plant (BM), HI and kernel related characters in dry salinity field (Luo et al. 2021). In cereals, salinity would mainly reduce the tiller number to decrease the total leaf area (Munns and Tester 2008). *QTn-5B* was stably detected in both ZX and XJ populations under S treatment, which could be a potential locus to improve salt tolerance. Furthermore, based on the wheat reference genome (IWGSC RefSeq v1.0), *QTn-5B* contained genes related to gibberellin-regulated family protein, ERD (early-responsive to dehydration stress) family protein, potassium transporter, calcium-binding protein and so on (Table S7).

In wheat, strongly influenced by chromosome positions, recombination rate was markedly higher toward the distal ends of the chromosomes than in the interstitial and proximal regions (Ramirez-Gonzalez et al. 2018). Most QTL were distributed on distal ends of both chromosome arms, which could increase the adaptive plasticity of wheat and was verified in present and previous studies. As a result of the allopolyploid nature of the wheat genome, quantitative variation for many agronomic traits is modulated by genetic interactions between multiple sets of homoeologs in A, B, and D subgenomes (Borrill et al. 2015). Moreover, QTL for seedling and grain yield traits associated with salt tolerance were detected in homoeologous regions (Ma et al. 2007; Quarrie et al. 2005; Xu et al. 2012; Xu et al. 2013). Based on 850 wheat RNA-sequencing data sets from different tissues, developmental stages and cultivars, it was found that about 70% of triads (A, B, and D homoeologs) showed balanced expression among homoeologs, whereas 30% showed nonbalanced expression patterns with higher or lower expression from a single homoeolog with respect to the other two (Ramirez-Gonzalez et al. 2018). In our results, QTL for root related traits (RL, RDW, TRL, TRT, LRL, LRT and LRSA) were found on the short arm distal ends of group-2 chromosomes but with distinctly different phenotypic variation explained (PVE). A typical example is that *QRl-2B* (3.5–4.5 cM, PVE = 46.43%) significantly explained more phenotypic variation than *QRl-2A* (33.5–47.5 cM, PVE = 5.29%) and *QRl-2D* (0–0.5 cM, PVE = 5.59%). Transcriptome analysis also demonstrated that syntenic triads in the balanced category were overrepresented in the low-recombination regions while homoeolog-dominant and homoeolog-suppressed triads were overrepresented toward the high-recombination distal ends of chromosomes (Ramirez-Gonzalez et al. 2018). This could be the reason why

three homoeologous genes were rarely detected at the same time in mapping studies and the possible homoeologous QTL explained different phenotypic variation.

Early, epistatic effect (*aa*) was found to play an important role in maize (Doebley and Stec 1995) and rice (Yu et al. 1997). Later, researchers discovered that in wheat *aa* was also significant for coleoptile growth (Rebetzke et al. 2007), water-soluble carbohydrates (Yang et al. 2007), plant height (Zhang et al. 2008), heading (Ashraf and Foolad 2013) and kernel morphometric traits (Prashant et al. 2012). Based on various studies (Azadi et al. 2015; Ilyas et al. 2020; Jahani et al. 2019; Ma et al. 2007; Nezhad et al. 2019; Quarrie et al. 2005; Villalta et al. 2007; Xu et al. 2012; Xu et al. 2013; Xue et al. 2009), the QTL detection would be inconsistent in a same genetic population, and the magnitude and direction of QTL effects, as well as LOD scores, could also be changed in different environments. Here, about a quarter of the QTL (39/158) were stable under both CK and S treatments, while about half of them (80/158) were observed only under CK conditions. QTL differed with environments, indicating significant QTL-by-environment effect (*at*) (Genc et al. 2013). Epistatic effect and QTL-by-environment effect had been reported for salt tolerance in wheat (Genc et al. 2013; Jahani et al. 2019; Masoudi et al. 2015; Nezhad et al. 2019; Xu et al. 2012; Xu et al. 2013). In the present study, although 94 pairs of *aa* were detected, only one for SWC was near to an additive QTL (*QSwc-2B.1*), which was consistent with other reports that the majority of the interacting loci had no significant main additive effect in wheat (Jahani et al. 2019; Reif et al. 2011), barley (Xu and Jia 2007), and rice (Li et al. 1997). Stable QTL across multiple environments are vital to MAS in wheat breeding. Hence, it is very necessary to figure out *at* effect under salinity stress in mapping studies. In this paper, QTL for TN (*QTn-2A* and *QTn-2D*), LN (*QLn-2D.2*), RL (*QRl-2B.2*), and SDW (*QSdw-4B*) were all stably detected under different treatments without significant *at* effect, and they could explain nearly 10% of the phenotypic variation. *QSh-4B.2* and *QRl-2B.1* discovered only under CK treatment could explain the maximum phenotypic variation (36.55% and 46.43%, respectively). Even though higher *at* effects were found at these two loci, they were very stable due to their validation in XJ population as well. Besides, *QSh-4B.2* should play a vital role during the growing period because it could also be detected at maturity stage. Consequently, the above loci should be on the useful list of MAS in salt tolerant wheat breeding.

Besides *aa* and *at*, genetic background (GB) also influences the QTL detection and MAS utilization in breeding. For examples, Cui et al. (2014) and Jahani et al. (2019) found that only a few of QTL were shared across 2–3 wheat GBs. Here, the parental lines of ZX and XJ population have a definite genetic relationship, while there is no direct or indirect relationship among the parents of ZX and LH populations. Verification experiments demonstrated that four stable major QTL were concurrently detected in both ZX and XJ populations and three QTL were shared by LH and ZX populations (Fig. 2). Since genetic positions on different genetic linkage maps were greatly different, same markers or consistent physical positions were the most credible information to decide if two QTL were the same one. With better wheat reference sequence and deeper mapping study, we would search out more reliable loci for MAS in salt-tolerant wheat breeding based on big data analysis.

In conclusion, this paper identified 158 stable additive QTL for 27 morphological and physiological traits at seedling stage of wheat. Among them, 19 QTL were detected with significant QTL × treatment effects (*at*), but none was found with epistatic effects (*aa*). About half of the QTL (78/158) were mapped in nine QTL clusters mainly on group-2 and – 5 chromosomes as well as 4B and 7D. Seven QTL intervals were further validated in the other two genetic populations. In addition, six SNPs linked to important QTL were successfully converted to KASP markers. Our results fully explored the genetic base of seedling traits (especially root system related traits) associated with salt tolerance in wheat, and will provide important information for MAS in salt tolerant wheat breeding.

Declarations

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Author Contributions

ZSL and QZ supervised the research. QZ and QLL designed the experiment. QLL and QZ performed the phenotypes of ZX RIL population. PH and QLL performed the phenotypes of XJ and LH population. QLL performed data analysis and QTL mapping, confirmed the QTL effects and wrote the manuscript. QZ also put forward many constructive suggestions and revised the manuscript. GTY, HWL, LQL, ZSW, and BL provided a lot of help in the phenotype identification and materials preparation. All authors read and approved the final manuscript.

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Tables

Due to technical limitations, table 1,2,3 is only available as a download in the Supplemental Files section.

Supplementary Information

Fig. S1 Detection of QTL for sNa on chromosome 2B and 5A using two mapping softwares. The solid lines in dark color on LOD graph denote the QTL detected with IciMapping 4.1; the dashed lines in light color denote the QTL detected with WinQTLCart 2.5.

Fig. S2 Validation of QTL for SWC on chromosome 6B in XJ population. The markers along the chromosomes in blue were the same SNPs in the common QTL intervals in ZX and XJ populations.

Fig. S3 The distribution of 158 QTL on all 21 wheat chromosomes. X-axis for each chromosome, Y-axis for the number of QTL.

Fig. S4 Genotyping of 24 RILs with the six developed KASP markers in this study. The genotypes obtained using KASP markers were coincided with the genotyping results on the Wheat55K SNP array.

Table S1 The nutrient solution contents in hydroponic culture

Table S2 Correlation analysis among three trials in ZX RIL population

*, ** and *** represent significant correlation determined by the Pearson correlation at 0.05, 0.01 and 0.001 probability levels, respectively.

Table S3 Correlation analysis among different seedling traits in ZX RIL population

The upper triangular matrix represents under CK treatment, the lower triangular matrix represents under S treatment. *, significant correlation determined by the Pearson correlation (P <0.05); **, significant correlation determined by the Pearson correlation (P <0.01).

Table S4 QTL for epistatic effect (*aa*) detected at seedling stage under CK and S treatments in ZX RIL population

PVE is short for phenotypic variation explained; Add represents the additive effect (*a*); AddbyAdd represents the epistatic effect (*aa*).

Table S5 Validated QTL detected in LH and XJ populations.

PVE is short for phenotypic variation explained; Add represents the additive effect; LeftCI and RightCI represent the left and right boundaries of the confidence interval. Positive additive effects indicate that alleles from Hanxuan 10 and Xiaoyan 54 enhance corresponding trait values, and negative additive effects indicate that alleles from Lumai 14 and Jing 411 enhance corresponding trait values in LH and XJ populations, respectively.

Table S6 The primer sequences of KASP markers developed in this study

Table S7 Possible candidate genes in three important QTL intervals based on the wheat reference genome (IWGSC RefSeq v1.0)

Figures

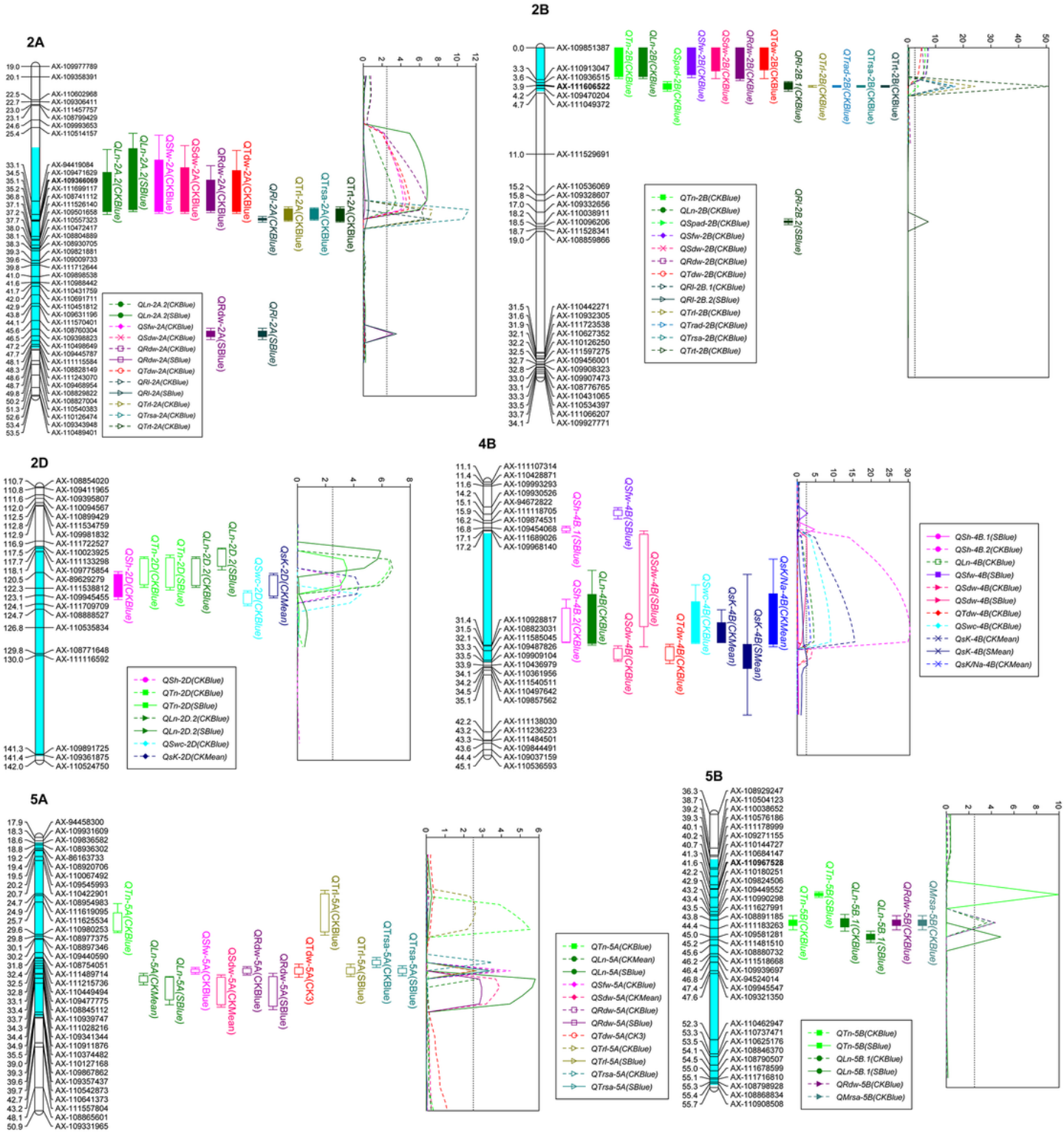

Figure 1

Six QTL clusters with LOD curves in this study and their involved QTL and SNP markers. The segments in cyan color of the six chromosomes present the intervals of the QTL clusters. The solid rectangles present that the alleles from XY60 increase the corresponding traits; the blank rectangles indicate that the alleles from ZM175 increase the corresponding traits. The solid lines on LOD graph denote the QTL detected

under salt treatment; the dashed lines denote the QTL detected under normal treatment. The SNPs in bold font were converted into KASP markers.

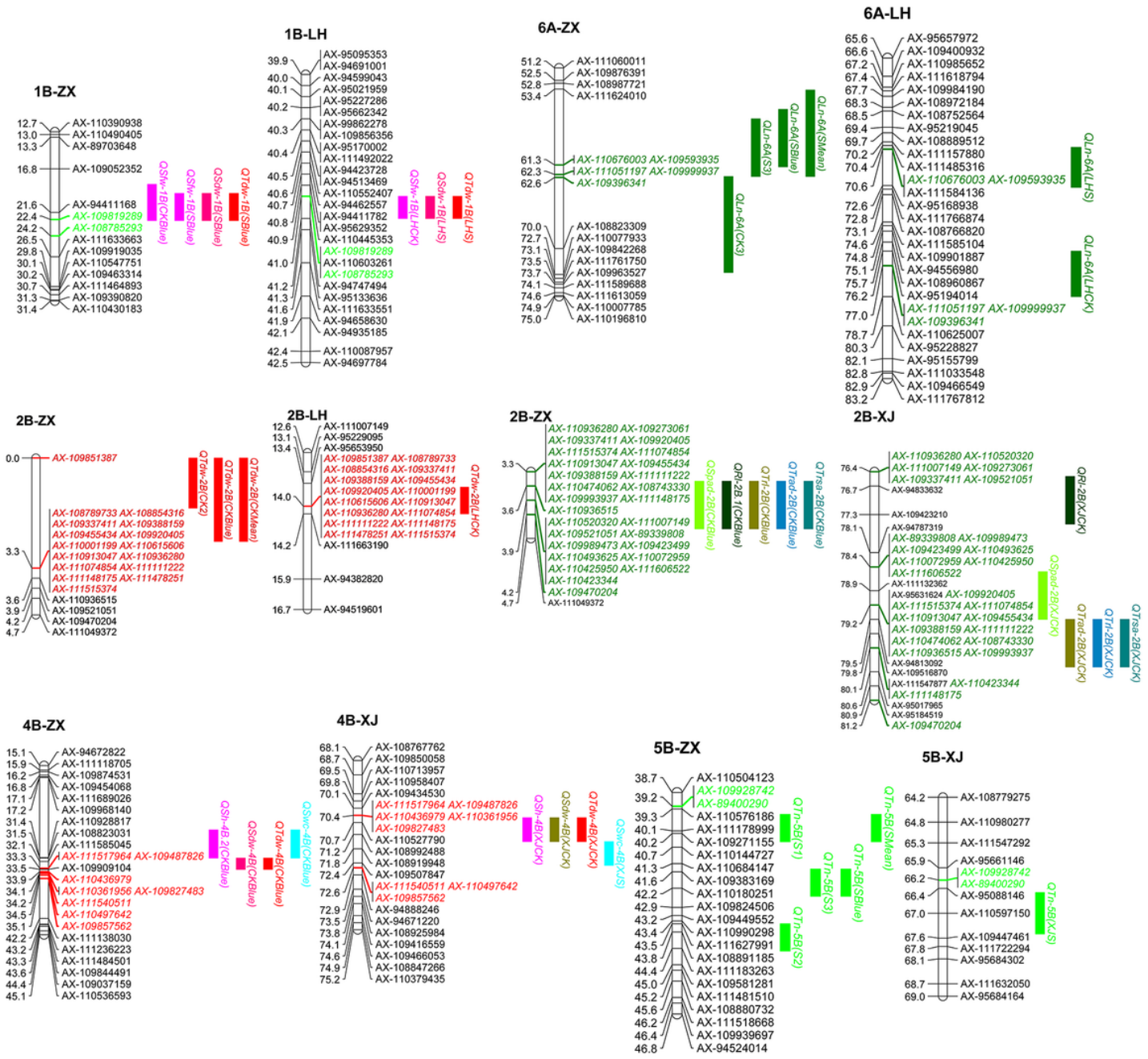

Figure 2

Validation of six QTL intervals in LH and XJ populations. Comparison of the common QTL detected in ZX and LH populations or in ZX and XJ populations. The colorful markers along the chromosomes were the same SNPs linked to the common QTL in two populations. The SNPs in bold font were converted into KASP markers.

Supplementary Files

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