Genome-wide association studies of ionomic and agronomic traits in USDA minicore collection of rice and comparative analyses of different mapping methods

Shuai Liu
Mississippi State University https://orcid.org/0000-0003-2244-2216
Hua Zhong
Wuhan University
Xiaoxi Meng
Mississippi State University
Tong Sun
Wuhan University
Yangsheng Li
Wuhan University
Shannon R. M. Pinson
USDA-ARS Dale Bumpers National Rice Research Center
Sam Chang
Mississippi State University
Zhaohua Peng (zp7@bch.msstate.edu)
https://orcid.org/0000-0001-6047-8434

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Abstract

Rice is an important human staple food vulnerable to heavy metal contamination due to its unique physiology and growth environment. High yield with low heavy metal contamination is a common but highly challenging goal for rice breeders worldwide due to lack of genetic knowledge. In this report, a comprehensive GWAS analyses for ionomic and agronomic traits based on 3,259,478 SNPs were performed using two univariate methods and two multivariate methods. Under the criterium p-value <1.53×10-8, 106, 47, and 97 QTLs were identified for ionomics in flooded environment, unflooded environment, and agronomic traits, respectively. Detailed analysis of the QTLs revealed that many of the identified QTLs are co-localized with the QTLs reported in prior ionomic and agronomic studies or posited near the genes with known functions in the related traits, suggesting that our GWAS analyses are reliable. Our results further showed that each of the four GWAS methods can identify unique as well as common QTLs. When univariate methods failed to identify QTLs for a trait, the multivariate methods frequently detected QTLs. However, when many QTLs were detected by univariate methods, the number of QTLs detected by multivariate methods were reduced in many cases. These analyses suggest that using multiple GWAS methods can complement each other in QTL identification and some methods may be more powerful with less false discovery rate. In addition, several candidate genes involved in ionomic and agronomic traits control were identified by sequence analysis of the QTL regions. This research provides novel insights into the genetic basis of both ionomic and agronomic variations in rice, establishing an important foundation for further studies on reducing heavy-metal contamination and improving crop yields.

Background

Rice is an important cereal which feeds more than half the world's population [1]. With the rapid expansion of global population, food security has become a highly challenging task. Meanwhile, anthropogenic activities such as mining, smelting, chemical engineering, energy related industry, and broad application of pesticides & fertilizers in agriculture has led to heavy metal contamination in soil, including Cadmium (Cd), Manganese (Mn), Nickel (Ni),], and metalloid Arsenic (As) [2]. Soil with excessive heavy metal elements represses plant germination and growth, resulting in a decrease of crop yield [3, 4]. Meanwhile, plants uptake the toxic heavy metal elements from contaminated soil and accumulate them in edible plant tissues, leading to food contamination.

The anaerobic growing conditions of flooded rice paddies and the unique physiology of the rice plant allow rice to take up some heavy metals from water and soils in a highly efficient manner and sequester it in different organs within the plants, including the grain consumed by humans. The arsenic concentration in a rice grain is roughly about 10 times higher than other crops grown in the same region even if the soil has no or limited anthropogenic contamination [5]. Rice has been reported to contribute substantially to inorganic and organic arsenic [6–8] intake by the human population in many regions of the world. Arsenic was ranked on the top of the US Agency for Toxic Substances and Disease Registry (ATSDR) Priority List of Hazardous Substances since 1997 (https://www.atsdr.cdc.gov/spl/index.html#2017spl). It

has also been listed as a toxic component by many other countries and treated as a critical contaminant during food safety inspection. Cadmium (Cd) is one of the most toxic heavy metals, and can easily reach the food chain due to strong assimilation by crops [9, 10]. Once absorbed, Cd is efficiently retained in the human body, may causing it to stay throughout the life span with an estimated half biology life between 6 to 38 years in kidney and between 4 to 19 years in the liver (ATSDR, 1999).

In contrast to heavy metals, many mineral elements are essential to humans but deficient in rice grains, for example, zinc, calcium, and iron [11]. To increase the concentrations of these minerals can improve the nutritional value of rice thus promoting human health for those using rice as the staple food. However, it is highly challenging to either increase the essential minerals or reducing the heavy metal due to lack of understanding of the genetic bases and molecular mechanisms of the related traits. Further, it is still poorly explored whether the concentration of mineral or heavy metal is associated with agronomic traits. Although there are multiple rice association mapping studies with specific minerals, heavy metals, and agronomic traits, respectively, these studies used either different mapping populations or different statistical analyses. Therefore, each of the studies reveals some but not all facets of the genetic bases of rice variations. Recent accessibility to comprehensive sequence data, and development of software facilitating use of more powerful statistical analytics, opens the opportunity for more comprehensive study and understanding of the genetic bases of these traits.

The USDA Rice Core Collection, containing about 10% of the whole NSGC (National Small Grains Collection) Rice Collection, was assembled by stratified random sampling method in 2002, which has been evaluated comprehensively for 25 characteristics and proven to be highly representative of the whole collection [12]. The Rice Mini-Core Collection contains approximately 10% of the Core Collection [13]. The grain mineral concentrations have been analyzed under flooded and unflooded growth conditions [14], and the agronomic traits have also been evaluated for the collection [15].

Biparental genetic mapping and Genome-wide-association-study (GWAS) are the two different tools for mapping Quantitative Trait Locus (QTL). GWAS involves studying a natural population thus reflecting historical recombination events, which is critical for crop improvement but cannot be revealed by studying the offspring of biparental crosses in linkage map [16]. GWAS has been applied successfully to a variety of plants, including Arabidopsis [17], maize [18, 19], barley [20], wheat [21], rice [22, 23], soybean [24], and cotton [25].

Univariate GWAS is a mapping method that has been successfully used for gene mapping in plants and animals. However, a large number of genes may not be detected (false negative QTLs) due to the confounding problems between population structure, kinship, and markers. The population structure causes genome-wide linkage disequilibrium between unlinked loci, which leads to statistical confounding in genome-wide association studies. Mixed models have been shown to deal well with the confounding effects of a large number of small effect loci in the diffusion background, but they do not always account for large effect loci [26]. Multivariate GWAS method considers the confounding problem between covariates and test marker to detect more QTLs with a lower threshold and false discovery rate. In recent

years, a large number of multivariate GWAS methods have been developed, including MLMM (multi-locus mixed-model) [26], FarmCPU (Fixed and random model Circulating Probability Unification) [27], mrMLM (multi-locus random-SNP-effect MLM) [28], FASTmrMLM (fast mrMLM) [29], FASTmrEMMA (fast multi-locus random-SNP-effect efficient mixed model analysis) [30], pLARmEB (polygenic-background-control-based least angle regression plus empirical Bayes) [31], pKWmEB (integration of Kruskal-Wallis test with empirical Bayes) [32], ISIS EM-BLASSO (iterative modified-sure independence screening expectation-maximization-Bayesian least absolute shrinkage and selection operator) [33], and GPWAS (Genome-Phenome Wide Association Study) [34]. The MLMM [26] uses forward-backward stepwise linear mixed-model regression, forward stepwise uses the most significant associated SNP as a new fixed-effect covariate (cofactor) and creates a new model until reaching a pre-specified maximum number of forward steps, backward stepwise means to remove least significant SNP and create a new smaller model until only one selected marker is left. Whereas, FarmCPU [27] performs marker tests with associated markers as covariates in a Fixed Effect Model (FEM), and then optimization on the associated covariate markers in a Random Effect Model separately. These multivariate GWAS methods were successfully applied to several different crop species, including cotton [35], rice [36, 37], foxtail millet [38], soybean [39, 40], maize [41, 42], and wheat [43, 44].

GWAS has been widely used for rice mapping research. However, most of the studies used univariate methods. The multivariate analysis methods and the deep DNA sequencing resources are still poorly explored. For example, Yan et al.(2014) [45] performed limited GWAS on agronomic traits using only 155 molecular markers (154 SSR makers and one indel marker). However, the related loci could not be precisely defined in the study due to limited number of markers. Recently, The resequencing data of the mini core has been deposited to SRA database https://www.ncbi.nlm.nih.gov/sra (Accession: PRJNA301661) [46], which opened opportunity for us to improve GWAS with higher density of genotypic data and evaluate the validity of different GWAS analysis methods. In this study, two univariate GWAS methods (GLM and MLM) and two multivariate GWAS methods (MLMM and FarmCPU) were employed to detect QTLs related to ionomic (As, Ca, Co, Cd, Cu, Fe, K, Mg, Mn, Mo, Ni, P, Rb, S, Sr, and Zn) and agronomic traits (amylose [AMYLOSE], awn type [AWNTYPE], flowering time [DAYSFLOWER], hull color [HULLCOLOR], hull cover [HULLCOVER], kernel length [KERNELLEN], kernel width [KERNELWID], kernel rate [KERNELRAT], kernel weight [KERNELWT], lodging [LODGING], panicle type [PANICLETYPE], plant height [PLANTHT], and plant type [PLANTTYPE]) simultaneously in USDA mini core accessions. Our results showed that these analyses successfully mapped most of the loci which have been shown to play essential roles in amylose and grain size control. Meanwhile, many novel loci involved in heavy metal, minerals, and agronomic traits control were discovered by using the multivariant GWAS methods. Furthermore, the correlation between different ionomic and agronomic traits were also analyzed. The study provided novel insights into the genetic basis of ionomic and agronomic variations in rice and possible correlations among various traits. The results will have critical reference value in fine mapping the related genetic loci and in guiding rice breeding.

Results And Discussion

Characteristics of SNPs

High-quality re-sequencing raw data of 191 accessions comprising the USDA Rice Mini Core, itself collected from 71 countries (Supplementary Table S1), was retrieved from NCBI SRA database (Accession: PRJNA301661) [46]. Genotyping of the 191 accessions were performed by GATK software. A total of 3,259,478 SNPs was obtained after filtration by minor allele frequencies (≥ 0.05) and integrity (≥ 0.4). Imputed SNPs, which were generated by Beagle 5.0 software [47], were used for further analyses. Distribution of these SNPs in the genome is summarized in Table 1 and Fig. 1a, and the overall SNP density in the genome was 114.51 (bp/SNP). The number of SNPs ranged from 212,238 to 375,296 across the twelve rice chromosomes. Chromosome 4 held the minimum marker density with 127.23 (bp/SNP), while chromosome 11 exhibited a maximum marker density with one SNP per 100.40 bp.

Table 1
Summary of the SNPs across 12 chromosomes of Oryza Sativa

Chromosome	Number of SNPs	Length of Chromosome (bp)	Density of SNP (bp/SNP)
1	375,296	43,270,923	115.30
2	301,111	35,937,250	119.35
3	294,312	36,413,819	123.73
4	279,049	35,502,694	127.23
5	253,001	29,958,434	118.41
6	287,238	31,248,787	108.79
7	253,651	29,697,621	117.08
8	261,070	28,443,022	108.95
9	212,238	23,012,720	108.43
10	222,521	23,207,287	104.29
11	289,053	29,021,106	100.40
12	230,938	27,531,856	119.22
Total	3,259,478	373,245,519	114.51

Population Structure and Linkage Disequilibrium

Admixture analysis divided the 191 accessions into four ancestries, including Indica (63 accessions), Aus (37 accessions), Temperate Japonica (28 accessions), and Tropical Japonica (31 accessions) under the best K model (K = 4) (Fig. 1b), which was determined by the lowest CV (cross-validation error) score

(Fig. 1c). Thirty-two accessions are classified as admixture (ADM) since the ratio from each single subpopulation is below 70%.

In order to reduce the amount of calculations, high-quality SNPs (SNPs integrity above 0.8) was selected to construct a maximum likelihood (ML) tree to illustrate the phylogenetic relationship of the 191 rice accessions (Fig. 1d). The population was divided into four subpopulations and the color for each clade was determined according to the Admixture analysis results. The relationship obtained from phylogenetic tree is in line with the Admixture analysis.

Principal component analysis (PCA) was performed based on the 3,259,478 SNPs. Four conceivable subpopulations were separated by PC1, PC2, and PC3. The first three principal components (PCs) explained over 50% of the genetic variation. The first PC separates Indica and Japonica subpopulations (35.70%), the second PC distinguishes the Aus and Indica varieties, while the third PC separates Temperate Japonica and Tropical Japonica varietal groups (Fig. 1e and 1f). Based on the results from the Admixture analysis, phylogenetic tree and PCA, the population was divided into four subgroups. In addition, the decay of LD with the physical distance between SNPs occurred at 191 kb ($r^2 = 0.2$) (Fig. 1g). Indica subpopulation exhibited the most rapid LD decay and Temperate Japonica showed the most extended LD.

Correlation of different traits

The correlation between grain ionomics in flooded environment and agronomic traits (Supplementary Fig. S1a), grain ionomics in unflooded environment and agronomic traits (Supplementary Fig. S1b), and between grain ionomics in flooded versus unflooded growth conditions (Supplementary Fig. S1c) were conducted. The results showed that days to flowering has strong correlation with Rb in flooded (0.53) and unflooded (0.57) (Supplementary Fig. S1a and S1b) environments. The accumulation of Cd, Mo, and Rb in rice grain in flooded environment and unflooded environment are correlated at $r^2 = 0.52$, 0.81, and 0.60, respectively (Supplementary Fig. S1c).

Genome-wide association study by univariate GWAS and multivariate GWAS

Sixteen grain ionomic traits (As, Ca, Co, Cd, Cu, Fe, K, Mg, Mn, Mo, Ni, P, Rb, S, Sr, and Zn) under flooded and unflooded conditions were evaluated with thee replications using ICP-MS method as reported [14]. Thirteen agronomic traits, including AMYLOSE, AWNTYPE, DAYSFLOWER, HULLCOLOR, HULLCOVER, KERNELLEN, KERNELWID, KERNELRAT, KERNELWT, LODGING, PANICLETYPE, PLANTHT, and PLANTTYPE [15], were collected and characterized as reported by Yan et al. [48, 49] and recorded using the methods described by Li et al [50–52]. All these traits were analyzed using two univariate GWAS (GLM and MLM) and two multivariate GWAS (MLMM and FarmCPU) methods to identify QTLs. A total of 106 significant QTLs (p-value $< 1.53 \times 10^{-8}$) were detected to be associated with 9 ionomic concentration (Cd, Co, Cu, K, Mo, Ni, Rb, Sr, and Zn) in rice grain under flooded condition, in which 63, 68, 17, and 44 significant QTLs were identified by GLM, MLM, MLMM, and FarmCPU, respectively (Fig. 2 and Supplementary Fig. S4b).

For Cd, twenty-eight significant QTLs were identified. Three of them located near published genes (CAL1 [53], OsHMA2 [54], rgMT [55]) which have shown to be related to Cd accumulation or resistance. Seven of them were also identified in previous mapping studies (Supplementary Table S2) and all of the seven QTLs were identified by univariate GWAS methods (GLM and MLM) but only two of the seven were also detected by multivariate methods (MLMM and FarmCPU), probably because the univariate methods were also used in reported studies. For Co, a total of eleven significant QTLs were detected. Two (one was identified by univariate methods and the other was detected by FarmCPU) of them co-located with previous reported QTLs. Nine of them were new QTLs discovered in the current study, MLMM method discovered 2 significant QTLs and FarmCPU method identified 7 QTLs, respectively. Three QTLs were detected to be significantly associated with K, one of which (only detected by FarmCPU) was also detected in previous studies [56]. For Zn, ten significant QTLs were identified, three of which co-located with previously reported loci [56–59]. Among them, one significant QTL posited around 1,8001,929 bp of Chromosome 7 was detected by both univariate and multivariate methods, which located near reported QTL qZN-7 [59]. The other two QTLs were detected by FarmCPU method only.

In the unflooded environment, only 47 QTLs were detected to be significantly associated with Cd, Fe, Mo, Ni, and Zn concentration. 29, 25, 10, and 20 significant QTLs were identified by GLM, MLM, MLMM, FarmCPU, respectively (Fig. 2 and Supplementary Fig. S4c). Twenty-three identified QTLs were related to Cd, one of which located near CAL1 gene [53], eight QTLs co-located with previous studies (Supplementary Table S2). Among the eight co-localization QTLs, five were detected by univariate methods and three were identified by multivariate methods. For Fe, seven significant QTLs were identified, two of which were also reported by previous studies [56, 57], and both were detected by FarmCPU only in the current study. From the result above, we noticed that for the traits that a large number of QTLs were identified using GLM and MLM methods, the numbers of QTLs identified by MLMM and FarmCPU were less as shown in the case of Cd and Mo. When GLM and MLM method failed to identify or only identified a few significant QTLs, QTLs were successfully identified by MLMM and FarmCPU methods as shown in the case of identifying QTLs for Co, Fe, K, and Zn concentration regulation (Supplementary Fig. S2), suggesting that the MLMM and FarmCPU method are sensitive and probably less likely to generate false positive QTLs. This observation is further confirmed when mining the key candidate genes controlling ionomic and agronomic traits as shown the section below. Interestingly, only 3 of the 106 (ionomic) QTLs identified in flooded growth condition were shared with the QTLs identified in unflooded condition, suggesting that flooding and unflooding growth condition may change the regulation of mineral and heavy metal uptake and accumulation in rice almost completely thus different pathways may be involved. The three QTLs (QTLs marked with an asterisk on Chromosome 5, 6, and 12; Fig. 2, Supplementary Fig. S2, and Supplementary Table S2) share by both growth condition were associated with Cd and Mo concentration regulation, indicating that the traits of these three QTLs were not impacted by water conditions. This result was consistent with the correlation analysis results of the ionomic traits that accumulation of Cd and Mo in rice grain in flooded and unflooded environment are correlated (Supplementary Fig. S1c) and the common QTLs for Cd and Mo under different irrigation conditions, respectively, are probably the genetic base for the correlation. In addition, two Cd concentration related

QTLs were detected in a proximate region on chromosome 2, one around the 25 M region and the other around the 24.9 M region. The first QTL is for flooded environment and the second is for unflooded environment. It is unknown if the encoded genes are similar. Furthermore, several loci were shown to be associated with more than one trait, indicating these QTLs may be pleiotropy. For example, the region around 15.5 Mb on chromosome 2 is associated with Cd and Mo (Fig. 2).

For agronomic traits, a total of 97 significant QTLs (p-value $< 1.53 \times 10^{-8}$) were detected for the thirteen agronomic traits described above except for KERNELWT and PLANTTYPE (Fig. 3, Supplementary Table S2). 39, 16, 29, and 50 significant QTLs were identified by GLM, MLM, MLMM, and FarmCPU, respectively (Supplementary Fig. S4a). Wax [60] and ALK [61] genes were shown to be significantly associated with amylose content, which is consistent with previous reports. Gain size is a key agronomic trait that strongly linked to yield and quality. Many QTLs have been reported associating with rice grain size, which is decomposed into grain length, width, and thickness (GS3 [62], GS5 [63], GW5 [64], GW8 [65], GL7 [66], TGW6 [67], etc.). In this study, four types of rice grain size-related traits included kernel length (KERNELLEN), kernel width (KERNELWID), kernel rate (KERNELRAT), and kernel weight (KERNELWT) were analyzed. A total of 13 QTLs were detected by univariate and multivariate GWAS methods. Among them, three were detected by univariate (GLM or MLM) GWAS methods and twelve of them were detected by multivariate (MLMM and FarmCPU) GWAS methods. 6, 2, and 5 of the 13 QTLs were found to be associated with KERNELLEN, KERNELWID, and KERNELRAT, respectively. No significant QTL was shown to be associated with KERNELWT. Remarkably, one QTL (Chromosome 3 position 16,733,441) was detected by all the four methods (Supplementary Fig. S3f). The QTL locates on gene GS3 [62], which is a major gene regulating grain size and organ size. This result confirmed the accuracy of the imputed SNP dataset and power of mapping QTLs with GWAS. It is worth to note that, five more significant SNPs were identified by FarmCPU and one of them located on chromosome 4 situated nearby the NAL1 gene, which has been shown to be related to rice yield [68]. For the trait of KERNELWID, two significant QTLs were only detected by MLMM method and other methods failed to identify candidate QTLs. One of the identified QTLs positioned around 5,364,561 bp of chromosome 5, which is about 0.56 kb apart from gene GW5 [69], which is known to control rice grain width (Supplementary Fig. S3g). These results demonstrated the power of GWAS, especially the power of the multivariate (MLMM and FarmCPU) GWAS methods.

Mining candidate genes of agronomic-related traits

Lodging and Plant height are both related to cell wall properties, which could impact rice yield. Appropriate plant height and the strong stem are required for stable production [70]. A cluster of SNPs around 33.4 Mb on chromosome 1 (Lodging: 33,010,693 to 33,975,764 with leading SNP at 33,469,251; Plant height: 33,181,529 to 33,730,067 with leading SNP at 33,363,796) is shown to be significantly associated with lodging and plant height (Supplementary Fig. S3j and S3k). Through LD block analysis, we defined a 72.37 kb blocks (33,458,683 – 33,531,049) containing 12 genes to be the candidate locus. Among these genes, OsPME6 (Os01g0788400) is annotated as pectin methylesterase 6, which is related to cell wall modification process. We further conducted blastP analysis with Arabidopsis thaliana and found that it shares high homology (E value = 3E-178) with Arabidopsis gene PME18 (AT1G11580)

(Supplementary Table S3). The expression of PME18 increased dramatically under hyper gravity stimulus. It is speculated that pectin esterases induced pectin demethylation of carboxyl groups which increased the rigidity of pectin gel in the cell wall through calcium bridges [71]. Therefore, it is worth to test if OsPME6 regulates rice lodging and height.

Flowering time is another important trait critical to rice production. Rice is a typical short-day (SD) flowering plant whose flowering is greatly affected by day length. A number of genes [72–75] have been reported to regulate rice flowering-time. In the current study, a total of three QTLs were significantly associated with the flowering time. Two of them were detected by FarmCPU exclusively on Chromosome 7 and 10. The other QTL on Chromosome 6 was detected with all the four different GWAS methods (Supplementary Fig. S3c). The haplotype analysis showed that this region only harbors 2 genes (OsPLL9 and OsPLL10). Among them, OsPLL9 (Os06g0583900) located 7.15 kb away from the leading SNP. This gene is a homolog of pectate lyase gene, which may play crucial roles during rice panicle development [76]. OsPLL9 is highly expressed in Stamen (one day before flowering), Palea (one day before flowering), and Panicle5 (heading stage) (Supplementary Fig. S5). Thus, OsPLL9 has the potential to be a candidate gene with a critical role in rice flowering.

Mining candidate genes of ionomic traits

28 and 23 significant QTLs were detected to be associated with Cd concentration in the flooded and unflooded environment, respectively. QTLs near CAL1 (Chr2:25,190,487 – 25,191,188) were associated with rice grain Cd accumulation in both flooded (Leading SNP; Chr2: 24,968,588) and unflooded condition (Leading SNP; Chr2: 25,143,071). CAL1 was annotated as a defensin-like protein, which could regulate Cd accumulation of rice leaves through translocating Cd from cytosol into extracellular spaces, but not rice grains [53]. We then further analyzed the genes around the QTLs and found there is an ABC transporter (Os01g0121700), its phosphorylation level was up-regulated under high Cd treatment (100 μM $CdCl_2 \cdot 2.5H_2O$) and it has been shown that the transporter reduces the concentration of Cd^{2+} through transporting PCs-Cd into vacuole [77]. Another QTL (Chr6: 29,733,715) was also showed strongly related to Cd concentration in rice grain. This QTL located near a known gene OsHMA2, which may decrease rice grain Cd concentration through suppressing the expression level of OsHMA2 [54]. In addition, significantly associated SNP (Chr11: 29,014,045) posited near rgMT gene, which was a metallothionein protein responded to the Cd stress in E. coli [55]. Comparing the QTLs detected in this study with previously reported studies, we found that over fifteen QTLs were co-localized with reported loci. The details were shown in Supplementary Table S2. Meanwhile, thirty-two new QTLs were identified.

A significant QTL was identified on Chromosome 1 around nucleotide 4,348,829 with p-value 3.37E-10 (MLM method). This QTL posited within a 9.97 kb block (Chr1: 4,345,517–4,355,489) containing only one candidate gene OsWRKY102 (Os01g0182700) (Fig. 4a and 4b). BlastP analysis showed that the OsWRKY102 (Os01g0182700) has high homology (1.00E-58) with Arabidopsis WRKY13 (AT3G39410) gene (Supplementary Table S4). WRKY13 activates the expression of gene PDR8 that encodes a Cd^{2+} extrusion pump, resulting in reducing Cd accumulation [78]. The expression profile from public data

showed that OsWRKY102 is intensively higher expressed in stem comparing to other tissues (Supplementary Fig. S6a). When treated with a high concentration of cadmium, the expression level of OsWRKY102 increased rapidly in both shoot and root (Supplementary Fig. S6b). Overall, the results suggested that OsWRKY102 responds at high-level cadmium treatment and regulates cadmium uptake and accumulation in rice. Another QTL (Chromosome 5 posited around 14,941,717) was identified in a flooded environment. Through LD analysis, we defined an 18.65 kb block (Chr5: 14,930,444 – 14,949,090) containing two genes, Os05g0321600 and Os05g0321900. Among them, Os05g0321900 (OsWRKY75) was annotated as DNA-binding WRKY domain-containing protein (Fig. 5a). BlastP analysis found that this gene shares high homology (4E-52) with WRKY55 (AT2G40740) of Arabidopsis thaliana, which regulates gold uptake and tolerance. Remarkably, one QTL (Chromosome 6 around position 11,906,590) was identified in both growth environments (Fig. 5a and 5b). OsMan07, is an Endo-Beta-Mannanase, only 24.82 kb away from the leading SNP and were also detected by the previous study [79]. BlastP analysis found this gene has a high similarity (6E-108) to Man3 (AT3G10890) gene of Arabidopsis thaliana (Supplementary Table S4). Overexpression of MAN3 enhanced Cd accumulation and tolerance, whereas loss-of-function of MAN3 led to decreased Cd accumulation and tolerance [80]. All the genes' expression patterns located in the haplotype region associated with Cd were showed in Supplementary Figure S7. Given that thirty-two new QTLs were identified in addition to precisely identified those loci reported in previous Cd regulation studies, this study should have important reference value for future research on Cd regulation in rice grain.

PIP2;6 has been reported to be involved in arsenic concentration control in rice. Although no SNP with Bonferroni-corrected significant thresholds -log 10(p) above 7.81 was discovered, there was an SNP peak with -log(p) around 6 on the chromosome 4 near the published gene PIP2;6 (Supplementary Fig. S2a), Suggesting that PIP2;6 is located near a significant QTL revealed by GWAS analysis

Comparison of univariate and multivariate GWAS Methods

Our results demonstrated that there was not a single method that was able to detect all the QTLs, suggesting that combining different methods can improve the power of QTL detection and increase the confidence in QTL identification. For example, GS3 gene was shown to be associated with grain length via both univariate GWAS and multivariate GWAS methods, the independent analysis results substantially increased the confidence for this QTL on grain size regulation. On the other hand, GW5 gene was detected to be related to grain width only by multivariate GWAS., No such a locus was detected by univariate method. In addition, it appeared that the multivariate methods were able to pinpoint the location of the QTLs more precisely on the chromosome compared with the univariate methods in many cases. As shown in Supplementary Fig. 3f, the peaks identified by univariate method were much broad than the peaks identified by multivariate methods. In addition, many QTLs were successfully identified by multivariate methods while univariate methods failed to identify significant QTLs. For example, GW5 was only detected by multivariate method to be associated with KERNELWID as mentioned above. Similarly, the Cu related QTLs in flooded conditions and Fe related QTLs in unflooded condition were also only detected by multivariate method. In addition, when a large number of QTLs were identified by univariate

method, the QTLs identified by multivariate method were substantially reduced. For example, over 29 QTLs for Cd were identified by univariate methods in flooded and unflooded environment but only six QTLs were identified by multivariate methods. These results suggest that multivariate methods are powerful and may be with low false discovery rate. However, the observation needs to be further confirmed.

Conclusion

In this study, a comprehensive GWAS analyses for ionomic and agronomic traits based on 3,259,478 SNPs were performed using two univariate methods and two multivariate methods. Under the criterium p-value $< 1.53 \times 10^{-8}$, 106, 47, and 97 QTLs were identified for ionomics in flooded environment, unflooded environment, and agronomic traits, respectively. Under flooded environment, 28, 11, 4, 3, 40, 3, 4, 3, and 10 significant QTLs were shown to be associated with Cd, Co, Cu, K, Mo, Ni, Rb, Sr, and Zn, respectively. In unflooded condition, 23, 7, 7, 7, and 3 significant QTLs were detected to be associated with Cd, Fe, Mo, Ni, and Zn, respectively. In addition, 18, 3, 5, 19, 6, 5, 2, 28, 4, and 7 significant QTLs were tightly associated with amylose concentration, flowering time, hull color, hull cover, kernel length, kernel rate, kernel width, lodging, panicle type, and plant height, respectively. Detailed analysis of the QTLs revealed that many of the identified QTLs are co-localized with the QTLs reported in prior ionomic and agronomic studies or posited near the genes with known functions in the related traits, suggesting that the results are reliable. Our results showed that each of the four GWAS methods can identify unique as well as common QTLs. When univariate methods failed to identify QTLs for a trait, the multivariate method frequently detected QTLs. However, when many QTLs were detected by univariate methods, the number of QTLs detected by multivariate methods were reduced in many cases. Our results suggest that using multiple GWAS methods can complement each other in QTL identification and some methods may be more powerful with less false discovery rate. Since we used multiple GWAS analysis methods and large-scale DNA sequencing results instead SSR markers for this study, more QTLs were identified, and the QTLs were defined to more precise regions compared with prior reports. Using the functional annotation results of the Arabidopsis thaliana orthologous, 3 plausible candidate genes (OsWRKY102, OsWRKY75, and OsMan07) are shown to be tightly associated with Cd concentration in rice (Supplementary Table S3). In addition, our results showed that OsPME6 or nearby gene may regulate plant height and OsPLL9 or its nearby gene may play a role in flowering time control.

Our comprehensive GWAS analysis of the ionomic and agronomic traits with large scale DNA sequencing data of the USDA mini core collection sets a foundation for further genetic and molecular biology studies on mineral, heavy metal, and agronomic trait regulation.

Methods

Plant Materials

Grain ionomic traits [14] and agronomic traits [15] of the mini core collection were the same as reported. Diverse rice accessions were grown over 2 years in Beaumont, Texas under both flooded (anaerobic) and unflooded (aerobic, flush irrigated) irrigation schemes. The planting, field management, and harvest methods were as reported [48–52]. The correlations of the traits were calculated by Pearson's correlation and visualized with R corrgram package [81]. The details of the samples are listed in (Supplementary Table S1).

Genotyping

In order to obtain high-quality sequencing data, the reads were first filtered by NGS QC Toolkit (v2.3.3) with default settings [82]. Then, the high-quality sequence was mapped to Nipponbare MSU7.0 genomic reference (http://rice.plantbiology.msu.edu/index.shtml, Release 7) with bwa program (version 0.7.17) using default parameters [83]. PCR duplicates were marked by Picard (version 2.18). Then, HaploypeCaller of GATK was used to identify SNPs. The raw SNPs were filtered by PLINK software with parameter '–maf 0.05 –geno 0.6 –snps-only'. Genotype imputation was performed for the remaining 3,259,478 SNPs with Beagle 5.0 [83] for further analysis.

Population structure, genetic analysis, and Linkage disequilibrium analysis

The raw SNP with integrity higher than 0.8 (181,448 SNPs) were extracted for estimating individual ancestries and constructing a phylogenetic tree. A PLINK software tool [84] was used to calculate the unlinked SNPs with parameter –indep-pairwise 50 10 0.2. Unlinked SNPs was submitted to ADMIXTURE [85] to assess the population structure with varying K from 2 to 10. Cross-validation error was calculated for each K, and the clustering model with the lowest cross-validation error (K = 4) was selected. Population structure was displayed using online software Pophelper (http://pophelper.com/). Each individual was assigned to one of the four subpopulations based on having ≥ 70% genetic ancestry derivation, the accessions that had < 70% ancestry from one specific subpopulation were assigned to a fifth group called 'Admix'. The matrix of pairwise genetic distance was used to construct phylogenetic trees using the software SNPhylo [86] with parameters set to 'default'. Principal component analysis (PCA) and kinship matrix (K matrix) were performed with 3,259,478 SNPs using default parameter by GAPIT [87]. The decay distance of LD (linkage disequilibrium) in each subpopulation and in the whole mini-core population were determined by software PopLDdecay [88].

Genome-Wide Association Study (GWAS)

GWAS was performed among 191 rice accessions derived from USDA mini-core collection with 3,259,478 high-quality SNPs. General Linear Model (GLM), Mixed Linear Model (MLM), Multi Locus Mixed Model (MLMM), and Fixed and random model Circulating Probability Unification (FarmCPU) were employed to evaluate the trait-SNP associations for grain ionomic and agronomic traits using the Genomic Association and Prediction Integrated Tool (GAPIT) [87]. Principal component analysis (PCA) was used as covariates to correct population structure due to subpopulations in the Mini Core. The genome-wide significant thresholds of the GWAS (p-value = 1.53×10^{-8}) was determined by 0.05/n (n is the number of

markers)[89] and a higher significant threshold was set as 3.06×10^{-9} (0.01/n) [90]. The Manhattan and QQ plots for GWAS were visualized using the R package CMplot (https://github.com/YinLiLin/R-CMplot). Leading SNPs of each significant SNPs cluster (in 200 kb) were selected to display the location of the QTLs.

Haplotype block estimation

Haplotype blocks containing at least two SNPs were calculated with all imputed SNP using the PLINK software [84] with the following parameters: ‘ –blocks no-pheno-req –blocks-max-kb 2000 –blocks-inform-frac 0.95 –blocks-strong-highci 0.98 –blocks-recomb-highci 0.9’. The haplotypic blocks of each significant SNP were determined by Confidence Intervals described by Gabriel [91]. The LD heatmap was visualized by software Haploview [92]. The annotated genes located in each haplotype block were extracted from RAP-DB (https://rapdb.dna.affrc.go.jp/) (Supplementary Table S5).

Gene expression data

The gene expression profile across 15 tissues (Endosperm, Callus, Seed, Radicle, Root, Plumule, Stem, Seedling, Shoot, Sheath, Leaf, Panicle, Spikelet, Lemma, and Stamen) was obtained from CREP (Collection of Rice Expression Profiles): http://crep.ncpgr.cn/crep-cgi/home.pl [93]. Gene expression data of rice plants treated with different cadmium concentration [94, 95] was adopted from TENOR (Transcriptome ENcyclopedia Of Rice): https://tenor.dna.affrc.go.jp/.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The genotype datasets analyzed during the current study are available in the NCBI SRA database (Accession: PRJNA301661), the phenotype traits analyzed are available in published article “Worldwide Genetic Diversity for Mineral Element Concentrations in Rice Grain” and website https://npgsweb.ars-grin.gov/gringlobal/descriptors.aspx

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Shuai Liu, and Zhuaohua Peng designed the work; Shuai Liu, Xiaoxi Meng, Tong Sun, and Yangsheng Li collected the dataset; Shuai Liu and Hua Zhong performed the analysis and finished the draft work; Each author substantively revised it.

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Figures

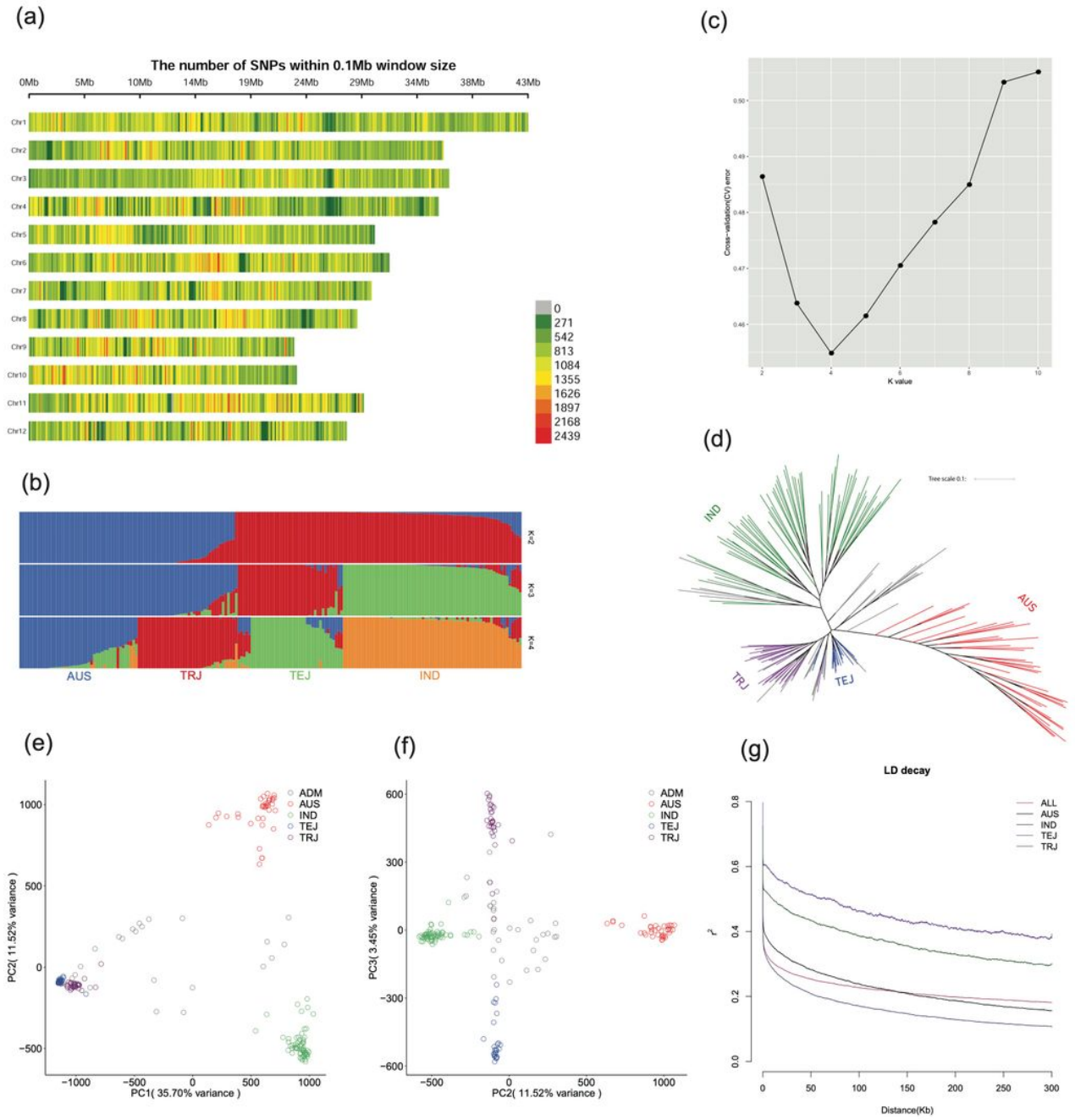

Figure 1

Sequence and structure analysis of USDA mini core collection. (a) Distribution of SNPs on the rice chromosomes. Number of SNPs per 0.1Mb window was shown as a color index (bottom right), (b) Ancestries analysis for each individual was inferred using admixture, (c) Cross-validation error (CV) score across different K value. The best K value (K=4) was chosen according to the lowest CV score for the admixture analysis, (d) Phylogenetic tree of 191 rice accessions. Green indicated Indica (IND) rice, Red indicated Aus (AUS) rice, Purple represented Tropical Japonica (TRJ) rice; Blue represented Temperate

Japonica (TEJ) rice, (e) PCA showing genetic variation in the rice accessions with first and second PCs, (f) PCA showing genetic variation in the rice accessions with second and third PCs, (g) Genome-wide average LD decay estimated from the whole population and each subpopulation.

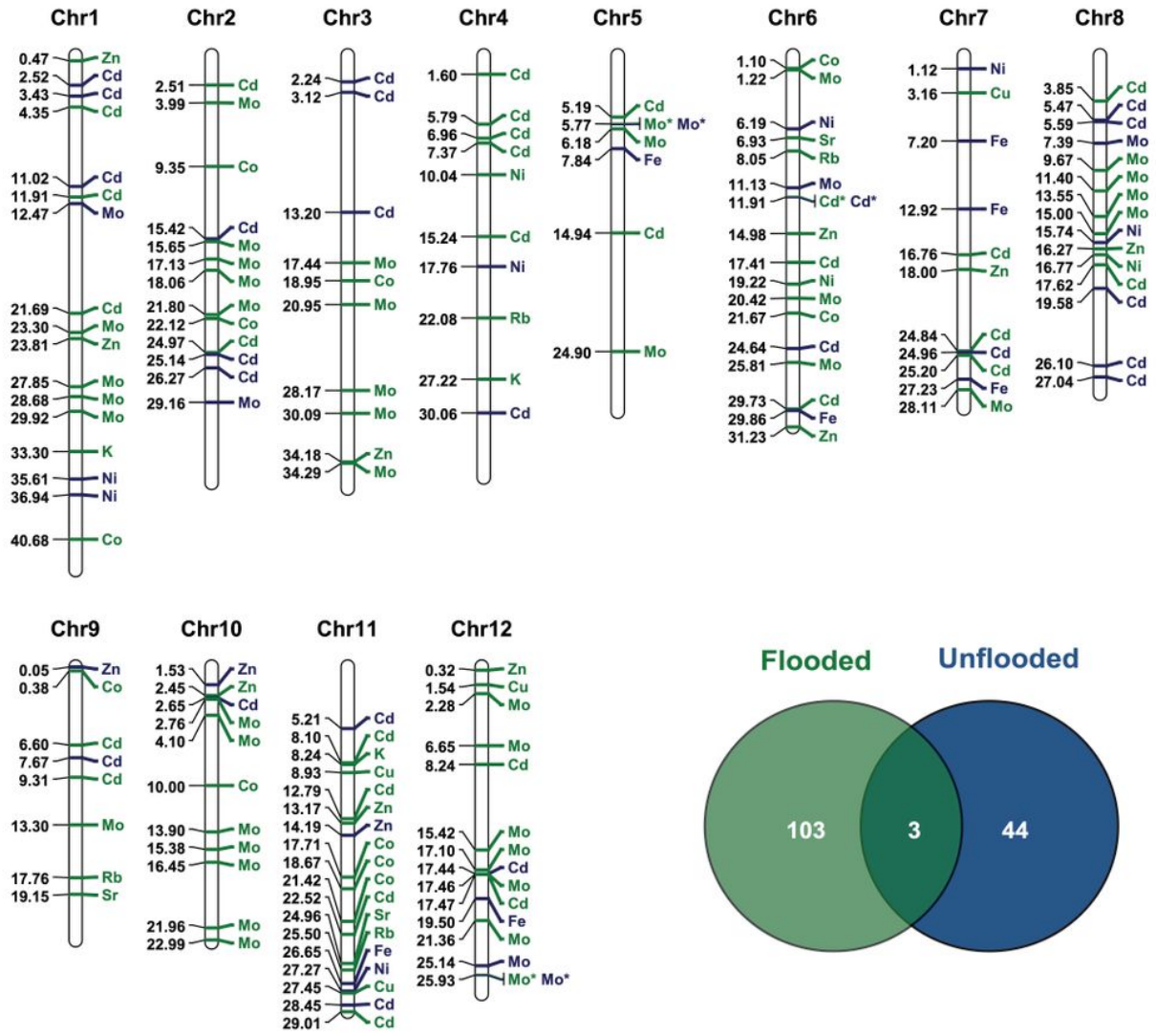

Figure 2

QTLs related to ionomic traits. (a) Distribution of significant QTLs for ionomic traits across the 12 chromosomes of rice under flooded and unflooded environment. Leading SNP was mapped to the chromosome to represent the QTLs' physical location. The physical position of each lead SNP was shown on the left side and the corresponding ionomic traits displayed on the right side. QTLs from different growth conditions were distinguished by different colors: green, flooded condition; blue, unflooded condition. An asterisk indicates the locus which was detected from both conditions, (b) The Venn diagram shows the numbers of overlapped loci within or between different conditions.

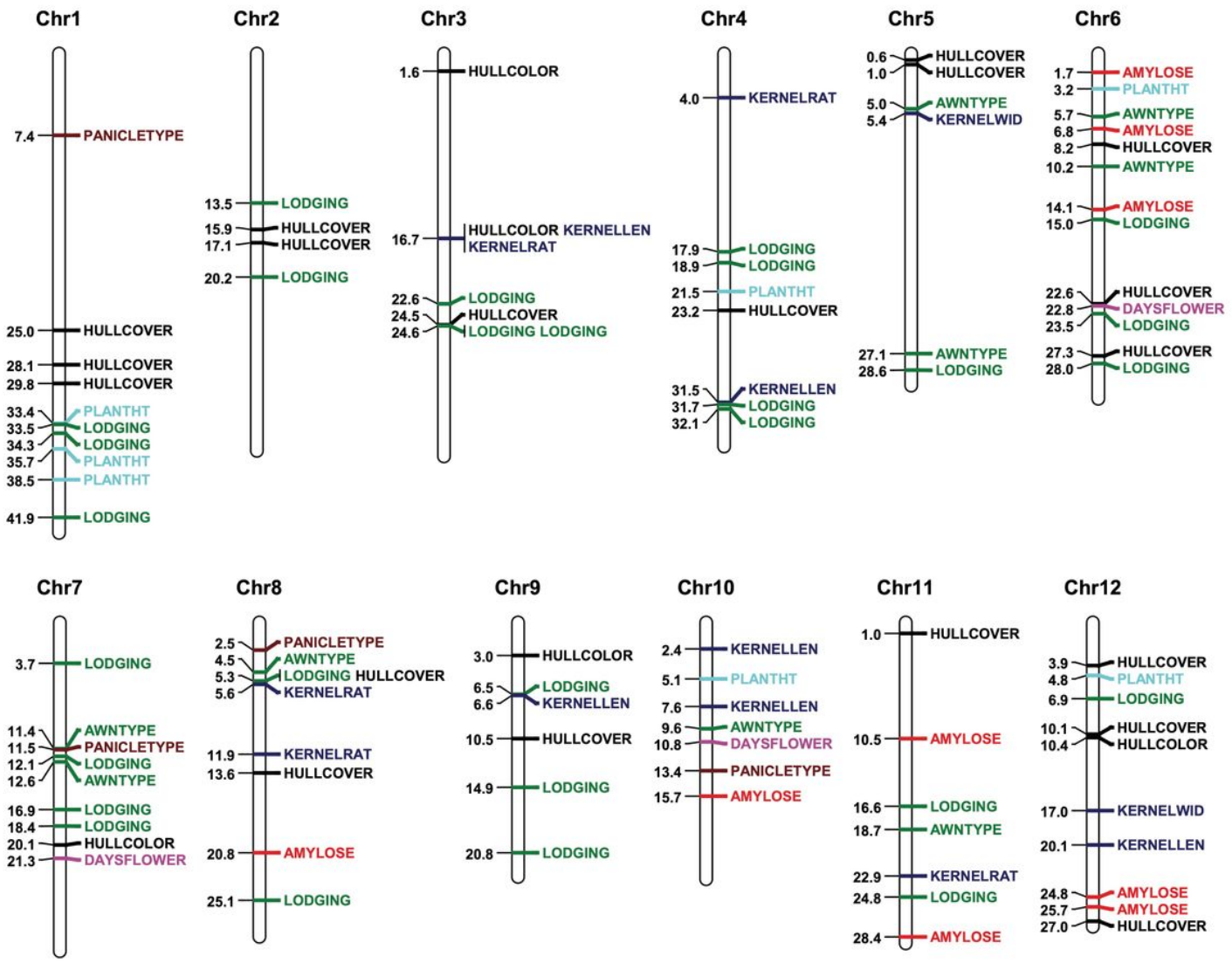

Figure 3

Distribution of significant QTLs for agronomic traits on 12 Chromosomes. Leading SNP was mapped to the chromosome to represent the QTLs' physical location. The physical position of each lead SNP was shown on the left side and the corresponding agronomic traits displayed on the right side. QTLs of different type of agronomic traits were distinguished by different colors: red, amylose; blue, grain size (kernel length, kernel width, and kernel rate); black, hull cover and hull color; purple, days to flower; brown, panicle type; green, lodging and awn type.

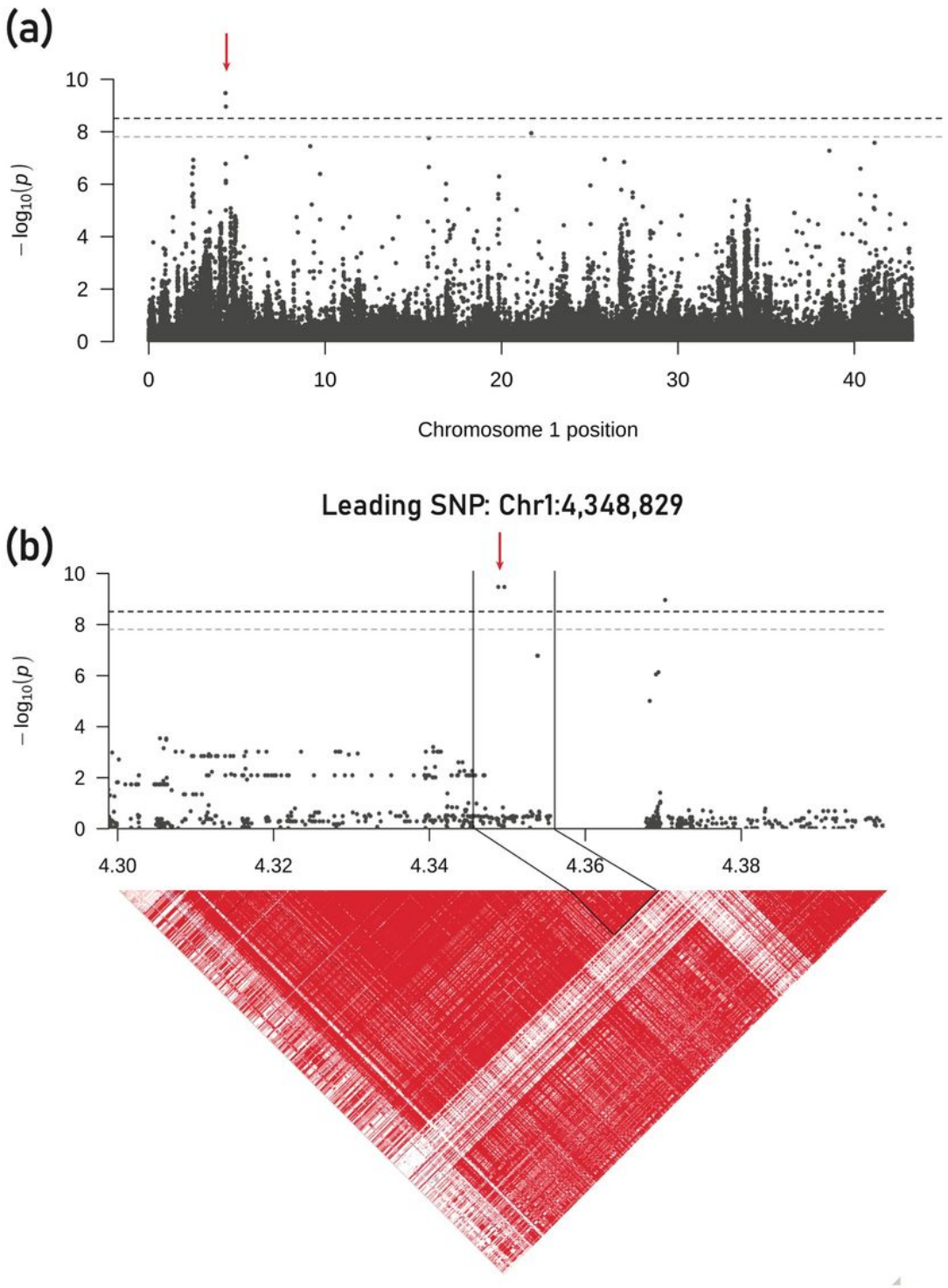

Figure 4

Identification of OsWRKY102 as a Cadmium concentration QTL in rice grain. (a) Genome-wide association signals on chromosome 1, (b) Genome-wide association signals in the region at 3.29 - 4.39 Mb on chromosome 1 and LD heatmap (bottom).

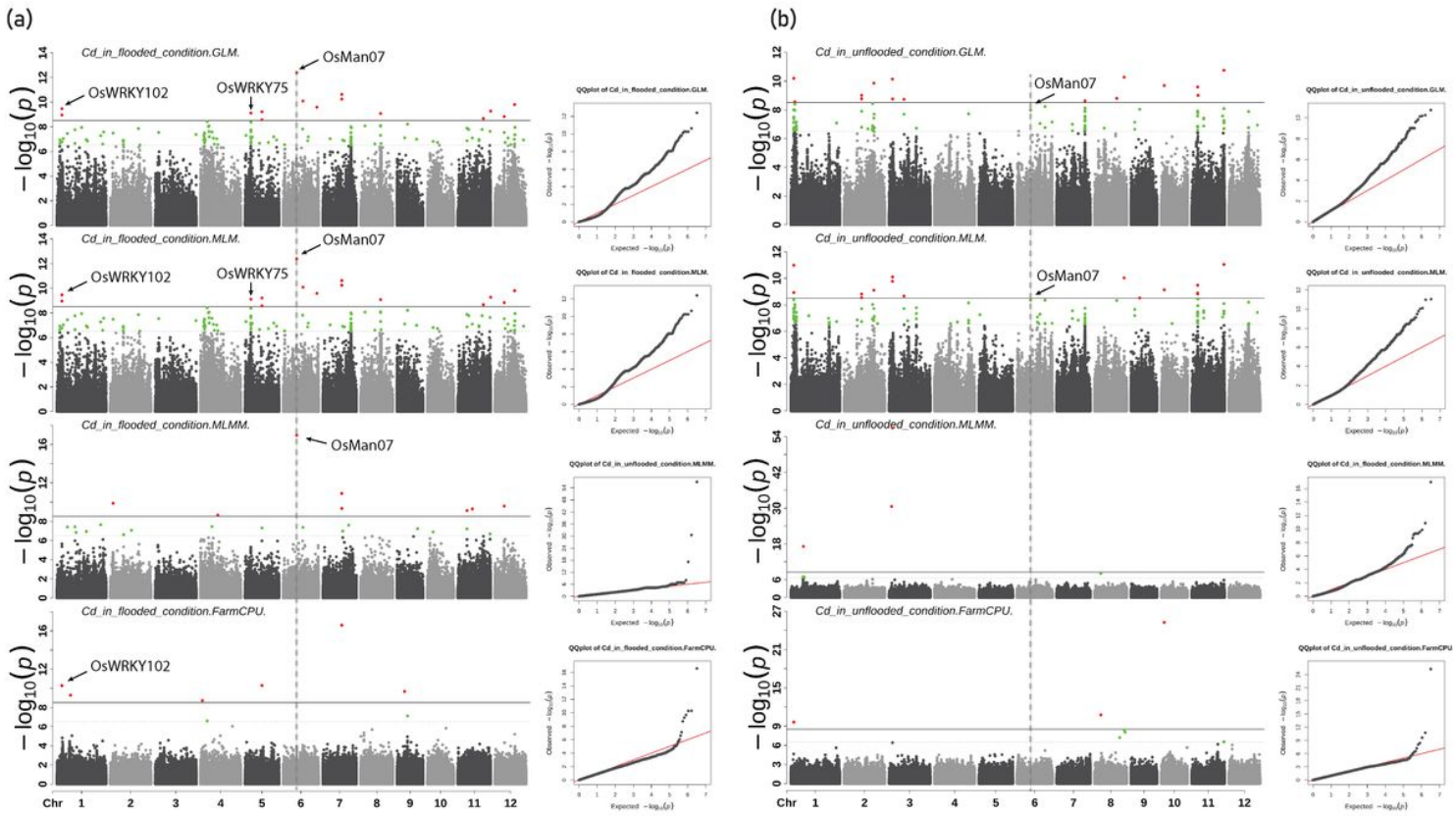

Figure 5

Genome-wide association analysis of Cd concentration with GLM, MLM, MLMM, and FarmCPU methods. (a) in flooded condition and, (b) unflooded condition. Quantile-quantile plot of each model. Black arrows indicated candidate genes. The horizontal dot grey line and green dots indicated the Bonferroni-corrected significance thresholds and SNPs at -log10(p) = 7.81. The horizontal solid grey line and red dots indicated the Bonferroni-corrected significance thresholds and SNPs at -log10(p) = 8.51. The vertical dash grey lines indicate the common QTL detected in flooded and unflooded condition.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryFigureS6.tif
- SupplementaryFigureS2.pdf
- SupplementaryTableS5.xlsx
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- SupplementaryTableS1.xlsx

- SupplementaryTableS4.xlsx
- SupplementaryFigureS4.tif
- SupplementaryFigureS7.tif
- SupplementaryTableS3.xlsx