Genetic basis of rice ionomic variation revealed by Genome-wide association studies

Meng Yang[a, 1], Kai Lu[a, 1], Fang-Jie Zhao[b], Weibo Xie[a], Priya Ramakrishna[c], Guangyuan Wang[a], Qingqing Du[a], Limin Liang[a], Cuiju Sun[a], Hu Zhao[a], Zhanyi Zhang[a], Xin-Yuan Huang[b], Wensheng Wang[a], Huaxia Dong[a], Jintao Hu[a], Luchang Ming[a], Yongzhong Xing[a], Gongwei Wang[a], Jinhua Xiao[a], David E. Salt[c], Xingming Lian[a, 2]

[a]National Key Laboratory of Crop Genetic Improvement and National Center of Plant Gene Research (Wuhan), Huazhong Agricultural University, Wuhan, China

[b]State Key Laboratory of Crop Genetics and Germplasm Enhancement, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, China

[c]Centre for Plant Integrative Biology, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, United Kingdom

[1]These authors contributed equally to this work

[2]Address correspondence to xmlian@mail.hzau.edu.cn

Abstract

Rice (*Oryza sativa*) is an important dietary source of both the essential micronutrients and the toxic trace elements to humans. The genetic basis underlying the variations in the mineral composition, the ionome, in rice remains largely unknown. Here, we describe a comprehensive study of the genetic architecture of the variation in rice ionome by performing genome-wide association study (GWAS) on the concentrations of 17 mineral elements in rice grain from a diverse panel of 529 accessions each genotyped at approximately 6.4 million SNPs. We identified 72 loci associated with natural ionomic variations, 32 of which are common across locations and 40 are common within location. We have identified candidate genes for 42 loci and provide evidence for the causal nature of three genes, *SKC1* for sodium, Os-*MOT1;1* for molybdenum and *Ghd7* for nitrogen. Comparison of rice GWA mapping data with *Arabidopsis thaliana* also identifies the well-known as well as new candidates with potential for further characterization. Our study provides new insights into the genetic basis of ionomic variations in rice, and serves as an important foundation for further studies on the genetic and molecular mechanisms controlling the rice ionome.

Introduction

Plants require at least 14 essential mineral nutrients and several beneficial elements for growth, development and resistance to biotic and abiotic stresses (Marschner and Marschner, 2012). Large quantities of macronutrient fertilizers [nitrogen (N), phosphorus (P), potassium (K)] are used in modern agriculture to increase crop yields, often resulting in adverse impacts on the environment (Withers and Lord, 2002). Increasing the use efficiencies of macronutrient fertilizers is critical for environmental quality and agricultural sustainability. Meanwhile, plants also take up non-essential and toxic elements from the soil, which may cause phytotoxicity or enter the food chain posing a

risk to human health (Clemens et al., 2002; Williams and Salt, 2009). Cultivated rice (*Oryza sativa* L.) is one of the most important crops that feed about half of the world's human population. For people who consume rice as the staple food, rice is a major dietary source of both the essential micronutrients [e.g. iron (Fe) and zinc (Zn)] and toxic elements [e.g. cadmium (Cd) and arsenic (As)] (White and Broadley, 2009; Zhao et al., 2010; Clemens and Ma, 2016). It has been estimated that up to two billion people worldwide suffer from Fe and Zn deficiencies, particularly in the populations with cereals as their staple food (White and Broadley, 2009; Swamy et al., 2016; Trijatmiko et al., 2016), and also a large number of populations poisoning with Cd and As especially for people in south Asian countries (Clemens and Ma, 2016; Chen et al., 2018). Enhancing the accumulation of essential micronutrients and reducing the concentrations of potentially toxic elements in rice grain are of fundamental importance for food quality and human health.

The composition of mineral nutrients and trace elements in plants, defined as the plant ionome (Lahner et al., 2003; Salt et al., 2008), is determined by the genetic, environmental and developmental factors as well as their interactions. Many genes responsible for the uptake, translocation and storage of mineral elements in plants have been identified. Based on studies using bi-parental populations, a large number of QTLs for mineral concentrations in rice have been reported (Lu et al., 2008; Garcia-Oliveira et al., 2009; Norton et al., 2010; Norton et al., 2012a; Du et al., 2013; Zhang et al., 2014; Mahender et al., 2016; Ohmori et al., 2016). To date, only a small number of the mineral QTLs have been finely mapped, leading to the identification of the causal genes, including Os-*HKT1;5* (or *SKC1*) for sodium (Na), Os-*HMA3* for Cd, *NRT1.1B* for N, and Os-*HMA4* for copper (Cu) (Ren et al., 2005; Ueno et al., 2010; Miyadate et al., 2011; Hu et al., 2015; Huang et al., 2016). However, the gene networks controlling mineral accumulation and homeostasis are complex and largely remain to be elucidated (Lahner et al., 2003). A pivotal task in plant ionomic research is to unravel the genetic basis

underlying the variations of the ionome among natural accessions of a plant species and crop cultivars. This knowledge is essential not only for understanding how plants adapt to their mineral environment, but also for potential exploitation of alleles for breeding crop cultivars with improved nutrient use efficiencies and better quality.

Genome-wide association study (GWAS) is a powerful tool to unravel the molecular basis for phenotypic diversity. It has the power to genetically map multiple traits and provides a complementary strategy to classical mapping using bi-parental synthetic recombinant populations for dissecting complex traits (Huang and Han, 2014). GWAS on the ionomic profiles of *Arabidopsis thaliana* accessions has led to the identifications of a number of loci as well as genes related to mineral accumulations, such as *HKT1;1* for Na (Baxter et al., 2010; Segura et al., 2012), *MOT1* for molybdenum (Mo) (Shen et al., 2012; Forsberg et al., 2015), *HMA3* for Cd (Chao et al., 2012) and *HAC1* for As (Chao et al., 2014). Rice landraces have evolved from their wild progenitors and show both high genotypic and phenotypic diversity (Huang et al., 2010). It is therefore possible to perform GWAS to identify the genetic basis of much of this phenotypic variation in rice. A large number of traits in rice, including numerous agronomic characters and metabolites, have been studied using GWAS (Huang et al., 2012a; Chen et al., 2014; Matsuda et al., 2015; Yano et al., 2016). Extensive heritable variation is known to exist in the rice grain ionome for 1763 rice accessions of diverse geographic and genetic origin (Pinson et al., 2015), and GWAS has been attempted on the concentrations of four minerals (As, Cu, Mo and Zn) in rice grain (Norton et al., 2014), eight elements [Zn, Fe, manganese (Mn), Cu, P, calcium (Ca), K and magnesium (Mg)] in brown rice (Nawaz et al., 2015) and on aluminum (Al) tolerance (Famoso et al., 2011). These studies show the promise of GWAS for ionomic traits, but they are limited by the low density of SNPs and bias caused by severe population structure inherent in the diversity panel containing both indica and japonica cultivars.

A common problem encountered in the studies of ionomic QTLs is the large effect of

the environment, especially soil properties and conditions that affect mineral supply and availability (Pinson et al., 2015; Huang and Salt, 2016), which may mask the genetic variation or render QTLs unreproducible. It is therefore important to conduct ionomic studies on rice diversity panels across different field conditions to help reveal both the generic and environment-specific QTLs. Here, we describe a comprehensive study of the rice ionome based on GWAS using 529 diverse rice accessions each genotyped at approximately 6.4 million SNPs. The rice diversity accessions were grown under five different field or nutrient conditions. Vegetative tissues at the heading and maturity stages and rice grains were analyzed for 17 mineral elements [N, P, K, Ca, Mg, Fe, Mn, Mo, boron (B), Cu, Zn, cobalt (Co), Na, Cd, As, lead (Pb) and chromium (Cr)] using high-throughput inductively-coupled plasma mass spectrometry (ICP-MS). We have identified a large numbers of significant marker-trait associations and multiple potentially causal candidate genes, three of which we have further validated. We have also compared rice and *A. thaliana* GWAS data and identified some conserved features of the genetic architecture underlying ionomic variations across plant taxa. The study provides new insights into the genetic basis of natural ionomic variations in cultivated rice and important information for comparative ionomic mapping in other plant species.

Results

Variations in the ionome among 529 cultivated rice accessions

To investigate the variation in the ionome in cultivated rice, a population of 529 cultivated rice accessions representing large variations in the geographical origin, the genetic diversity and the usefulness for rice improvement, was used in this study (Supplemental Data set 1). As described in our previous works (Chen et al., 2014; Zhao et al., 2014; Xie et al., 2015), this population has been sequenced using the Illumina Hiseq 2000 system, generating a total of 6.7 billion reads. We included the sequences from 950 rice varieties generated by Huang et al. (2012a) and selected SNPs with

missing rates of less than 20%. We obtained a total of 6,428,770 SNPs with a missing data rate (SNP call rate) of approximately 0.38% for use in the present study. Comparisons between the imputed genotypes and relevant high-quality genome sequences in the database as well as our array-based genotypes showed a high accuracy (>99%) of the imputed genotypes (Xie et al., 2015). The SNPs and imputed genotypes can be queried at RiceVarMap; http://ricevarmap.ncpgr.cn (Zhao et al., 2014). Analysis of the sequence data shows that the 529 accessions include 295 *indica*, 156 *japonica*, 46 *aus* and 32 intermediate. Among the 295 *indica* accessions, 98, 105 and 92 accessions are further grouped into *indica* I (IndI), *indica* II (IndII) and *indica* intermediate, respectively. The 156 *japonica* accessions are further grouped into temperate *japonica* (TeJ, 91 accessions), tropical *japonica* (TrJ, 44 accessions) and *japonica* intermediate (21 accessions) (Supplemental Data set 1).

The ionome is influenced by genetic and environmental variations, and the development stage (Salt et al., 2008). N and P are two major nutrients that have large impacts on plant growth and development. In the present study, we first investigated the variation in the rice ionome among 529 accessions grown in three adjacent paddy fields in Wuhan varying in the status of N and P supply, including a normal field (NF) receiving standard N and P fertilization, a low N field (LN) and a low P field (LP) where either N or P fertilizers have been withdrawn for the last decade. The ionomic compositions (N, P, K, Ca, Mg, Fe, Mn, Mo, B, Cu, Zn, Co, Na, Cd, As, Pb and Cr) of the shoots at the heading stage, straw and brown rice at maturity were determined.

We found that the scale of ionomic variation depends on both the element and the plant tissue, with the essential macronutrients [N, P, K, Ca, Mg, relative standard deviations (RSD) varying between 10 – 38%] showing smaller variations than micronutrients (Fe, Zn, Cu, Mn, B, RDS varying between 20 – 191%) or nonessential trace elements (Cd, As, Co, Cr, Pb, RSD varying between 28 – 196%), and the reproductive tissue (brown rice) showing smaller variations than the vegetative tissues

(shoots or straws) (Supplemental Figure 1 and Supplemental Table 1). The broad-sense heritability (H^2) of the 17 elements, averaged across different field conditions and tissue types, ranged from 0.31 to 0.88 (Supplemental Table 2), consistent with previous reports that a significant portion of ionomic variation has a genetic basis (Pinson et al., 2015). Particularly, elements such as N, P, K, Mg, Mo, Zn, As and Cd showed high average H^2 (≥0.68). The ionomic compositions were also influenced by N and P treatments. Low N resulted in significant decreases in the concentrations of most elements, whilst low P also led to lower concentrations of some elements (e.g. N, Na, Fe, As, Cd and Pb). In general, LN had larger impact on rice ionome compared to LP as revealed by multivariate analyses. Principal component analysis (PCA) based on shoot or straw ionome revealed a reasonable separation of accessions between NF and LN field conditions but not between NF and LP fields (Supplemental Figure 2). Similar to PCA results, hierarchical clustering analysis based on shoot or straw ionome showed that accessions grown in NF or LN fields were generally clustered together, while such clustering was not observed between accessions from NF and LP fields (Supplemental Figure 3). *Indica* and *japonica* are two main subspecies of cultivated rice showing significant diversity in genetic architecture. In general, *japonica* accessions accumulated significantly higher levels of most elements than *indica* accessions (Supplemental Table 3). Median concentrations of Na, Mn, Co, Zn, and Mo in *japonica* accessions were consistently higher than those of *indica* accessions in all three tissues under three field conditions, with Mo concentrations showing the largest difference (1.4 – 3.9 times) (Supplemental Table 3). In contrast, median Cd concentration in brown rice of *japonica* accessions was considerably lower than that of *indica* accessions, which is in agreement with previous reports (Arao and Ae, 2003; Uraguchi and Fujiwara, 2013; Pinson et al., 2015). The fact that the median shoot Cd concentration at the heading stage was larger in *japonica* than in *indica* suggests a lower translocation of Cd from the shoots to the grains in the former.

Fig. 1 summarizes the influence of field condition, plant tissue and subspecies

(*indica* versus *japonica*) on the ionome. The type of plant tissue has the most distinctive effect on the ionome, with N, P, Mo, Cu and Cr being preferentially distributed to brown rice and forming a cluster, and the other 12 elements in a second cluster showing an opposite pattern. The brown rice ionome appears to be separated according to the subspecies, whereas the ionomes of shoot and straw are separated by the N and P status of the fields.

To investigate further the effects of environment on the rice ionome, we grew the rice population at Youxian, Hunan province, for two consecutive years. The field site at Youxian had a lower soil pH and a higher Cd concentration than the soil at Wuhan (Supplemental Table 4). Ionomic analysis was conducted on both the vegetative tissue and rice grain (dehusked) harvested at crop maturity. Similar to the results from the trials at Wuhan, most mineral elements showed relatively high H^2 across the two years at Youxian (Supplemental Table 5). The differences in the mineral concentrations between *indica* and *japonica* subpopulations were also generally consistent between Youxian and Wuhan sites (Supplemental Tables 3 and 6).

Genetic basis of variations in the rice ionome

Based on the ionomic and sequence data of 529 rice accessions, we performed GWAS in in the *indica* and *japonica* subpopulations separately, using both simple linear regression (LR) and a linear mixed model (LMM). Because LMM results in fewer false positives (Huang et al., 2010), the GWAS results based on LMM are presented here. *P* values of 1.8×10^{-6} and 4.1×10^{-6} were set as the significance thresholds for *indica* and *japonica* respectively, after Bonferroni correction. Before performing GWAS, the Box-Cox procedure was used to normalize the elemental traits which were non-normally distributed (Supplemental Figures 4 to 7). For each element, a series of SNPs in a defined region with *P* values lower than the threshold were detected. Since the median distances of linkage disequilibrium (LD) decay in *indica* and *japonica* of this rice population had

been revealed to be 93 kb and 171 kb, respectively (Xie et al., 2015), the significant SNPs within a 300 kb region are considered to represent a locus, and the SNP with the lowest *P* value in a locus is defined as the SNP in closest linkage to the causal gene. Based on this criterion, a total of 41 loci (or associations) that were detected in at least two field conditions for the same tissue of the same subpopulation at Wuhan and 14 loci were in the two consecutive years at Youxian (Supplemental Data sets 2 and 3). Moreover, 32 loci were scanned across the locations of Wuhan and Youxian (Supplemental Data set 4) and 2 loci were detected in all five field trials. Out of the total 87 loci detected, 72 were unique (Figures 2 and 3, and Supplemental Figure 8). Of the 17 mineral elements analyzed, all 17 elements had at least one GWAS locus and 9 elements had at least 4 loci.

Functional interpretations of the ionome GWAS results

The ionomic loci identified by GWAS provide important clues for understanding the genetic architecture of the observed variations in the rice ionome. To identify candidate genes responsible for each ionomic locus, we extracted all genes within 300 kb of the most significant SNPs and considered their annotations, functions of homologous genes and distance from the peak SNPs (Supplemental Data set 6 and Supplemental Figure 9). By applying this approach, we obtained a list of genes that represent plausible candidates for the causal gene for each of the loci controlling elemental concentrations in rice (Supplemental Data set 7).

We chose 2 loci for further investigation with the aim of identifying the causal genes. We found that Os-*HKT1;5* is close (~14 kb upstream) to the most significant SNP (sf0111478828) of a Na related locus, which was detected at both Wuhan and Youxian locations (Figures 4A and 4C, and Supplemental Data set 7**)**. Os-*HKT1;5* is a Na transporter which was previously isolated in rice by map-based cloning using parents of Nona Bokra (a salt-tolerant *indica* variety) and Koshihikari (a susceptible elite *japonica*

variety) (Ren et al., 2005), suggesting that allelic variation in Os-*HKT1;5* explains the phenotypic variation in salt tolerance in the two rice varieties. Furthermore, the study showed that the phenotypic variation is caused by the difference in the transport activity of Os-HKT1;5 (Ren et al., 2005), not by the difference in the Os-*HKT1;5* expression level. Based on our sequence data, we found 9 SNPs which result in 9 amino-acid changes in the coding sequence of Os-HKT1;5 (Figure 3C), including the four amino acids which were suggested to be responsible for the functional difference between the Nona Bokra and Koshihikari alleles at Os-*HKT1;5* (Ren et al., 2005). We investigated the linkage relationship between these 9 SNPs and the most significant SNP at this locus. Interestingly, only one SNP (sf0111461701) is in strong linkage with the peak SNP (Figure 3D). This SNP displays a substitution at the 184th amino acid of Os-HKT1;5 (H184R) with the variation occurring mainly in the *indica* subspecies (Figure 3E). The two allelic groups separated by the base type of this SNP showed significant difference in the straw Na concentration in all three field conditions, with the T allele (corresponding to H at the 184th position of amino acid) containing lower Na than the C allele (corresponding to R at the 184th position of amino acid) (Figures 3F to 3J). Because the above 9 SNPs are not closely linked with each other, there are likely to be more than two types of Os-*HKT1;5* alleles. According to these 9 SNPs, there are 21 haplotypes of Os-HKT1;5 in all 1479 accessions [529 accessions used in this study and 950 accessions sequenced by Huang et al. (2012a); Supplemental Figure 10]. Both the haplotypes of *HKT1;5*^{NB} (Os-*HKT1;5* from Nona Bokra, designated as Hap2) and *HKT1;5*^{K} (Os-*HKT1;5* from Koshihikari, designated as Hap3) were found in the accessions (Supplemental Figure 11). In fact, only six haplotypes (from Hap1 to Hap6) exist in more than 10 accessions among the 1479 accessions. We analyzed the Na concentrations of the 6 haplotypes in our collection. We found that the Hap2, which is the only haplotype representing “H” amino acid at the 184th position, showed the lowest Na concentration in straws compared to other haplotypes in each subpopulation (Figure 5). These results

suggested that Hap2 is the strongest allele in restricting Na accumulation in the shoots and in Na tolerance. It has been suggested that Os-*HKT1;5* functions in the roots to recycle Na out of the xylem to limit the translocation of Na to the shoot. Loss or reduced function of Os-*HKT1;5* therefore leads to increased shoot Na concentration (Horie et al., 2009). Taken together, our results indicate that H184R is likely the key substitution that causes functional variation in Os-*HKT1;5*. Unlike its homologous gene *HKT1;1* in *Arabidopsis thaliana* (Baxter et al., 2010), Hap2 of Os-*HKT1;5* shows no obvious difference in the geographical distribution from other haplotypes (Supplemental Figure 11), which may be caused by the fact that rice is domesticated and therefore the link between genotype and the environment is now lost. Interestingly, Os-*HKT1;5* is relatively conserved in *japonica*, with most accessions (93.5%) possessing the weak allele Hap3 (Supplemental Figure 10). Only one *japonica* accession has the Hap2 allele. Therefore, the Hap2 allele of Os-*HKT1;5* has the potential to enhance salt tolerance of *japonica*, as well as *indica* cultivars without this allele. Furthermore, IndII accessions contained significantly lower Na concentrations in the shoots compared to other subpopulations with the same Os-*HKT1;5* haplotype (Supplemental Figure 12), regardless whether the Os-*HKT1;5* haplotype is strong or weak, suggesting that the IndII accessions harbor additional genes involved in regulating Na accumulation.

Because SNP sf0800123053 is strongly associated with Mo concentration (Figures 6A and 6B), we investigated the causal gene for the locus. SNP sf0800123053 is located 37 kb from LOC_Os08g01120 (encoding a putative sulfate transporter and named Os-MOT1;1), which is the closest orthologue of At-*MOT1* (a major Mo transporter in *Arabidopsis*) (Tomatsu et al., 2007; Baxter et al., 2008). GUS staining driven by the Os-*MOT1;1* promoter revealed that Os-*MOT1;1* was mainly expressed in the lateral roots, with weak expression in leaf blade, leaf sheath, culm, node and grain (Supplemental Figure 13). We obtained a knock-down mutant of Os-*MOT1;1* and found that the mutant accumulated significantly lower concentrations of Mo in both roots and shoots than

wild-type plants (Supplemental Figure 14), supporting the suggestion that Os-*MOT1;1* is the causal gene underlying this Mo locus. Within this locus, significant SNPs were found almost exclusively in *indica* subpopulations. One of these SNPs, sf0800085089, was found to land directly on the promoter region of Os-*MOT1;1* (Figure 6C). Moreover, accessions separated by SNP sf0800085089 in *indica* (hereafter called Ind-C and Ind-T) showed significant difference in straw Mo concentrations in all five field trials (Figures 6E to 6I), suggesting that Os-*MOT1;1* contributes to the variation of Mo concentration in *indica*. The nucleotide diversity of Os-*MOT1;1* genomic sequence in *indica* was then analyzed. Several SNPs or INDELs were found to show a close linkage to SNP sf0800085089 (r^2>0.65) (Figure 6D). One INDEL locating at the 5` UTR of Os-*MOT1;1* with a 10 bp insertion in the group Ind-C (higher Mo group) and one SNP locating at the ORF of Os-*MOT1;1* resulting in one amino-acid change were found and confirmed by fully sequencing 20 randomly selected *indica* accessions (Figure 5C). Additionally, the fully sequenced data revealed a number of new variation sites in the promoter region including two insertions of 999 bp and 226 bp fragments, and some deletions of short fragments (Figures 6J, 6K and Supplemental Figure 15), but no additional variation was found in the Os-*MOT1;1* coding sequence (Supplemental Figure 16). To test whether the variations in the promoter region affect the expression level of Os-*MOT1;1* among the *indica* accessions, the Os-*MOT1;1* transcript levels in the roots of 10 accessions each of Ind-C and Ind-T groups were determined under both Mo sufficient and deficient conditions. The Ind-C group with higher Mo concentration had significantly higher levels of Os-*MOT1;1* transcript than the Ind-T group under both conditions (Figure 6L and 6M). At least four variants were found in the Os-MOT1;1 protein among the 529 rice accessions (Figure 7A). To investigate whether there are functional differences among these variants, we over-expressed the four Os-MOT1;1 types in rice plants (*O. sativa* L. ssp. *japonica* cv. Zhonghua 11) and selected 6 transgenic lines for each Os-MOT1;1 type, 3 with relatively high expression and 3 with relatively low expression, for further study

(Figure 7B). We found that overexpression of Os-*MOT1;1* increased Mo concentration in both roots and shoots in an expression level dependent manner (Figures 7C and 7D). The relationships between the expression level and root Mo concentration were similar among the four types of Os-*MOT1;1* over-expressors (Figure 7E). For shoot Mo concentration, the type2 over-expressing plants appeared to be more effective than the other three types, but there was no significant difference among types 1, 3 and 4, the latter two types corresponding to the accessions of Ind-T and Ind-C, respectively (Figure 7F). Taken together, our evidence is consistent with Os-*MOT1;1* being the causal gene for the Mo locus with the lead SNP sf0800123053, and that the variation in Mo concentration is caused by the allelic variation in the promoter region leading to variable expression of Os-*MOT1;1*.

Comparative GWAS for ionomic traits between rice and *A. thaliana*

It has been suggested that natural ionomic variation in different species tends to be controlled by genes from the same gene families (Huang and Salt, 2016). Over recent years, GWAS has been extensively performed in *A. thaliana* and several significant ionomic loci have been identified using a set of ~349 wild accessions (Baxter et al., 2010; Chao et al., 2012; Chao et al., 2014; Forsberg et al., 2015). Comparing the rice ionomic GWAS data described here with that previously obtained using *A. thaliana* should allow us to explore more systematically to what degree the genetic architecture controlling ionomic variation is conserved across taxa between rice and *A. thaliana*. This comparative GWAS approach could also provide further evidence in support of candidate genes. We reran the GWAS using the publically available leaf ionomic data from the set of 349 *A. thaliana* accessions with an updated fully imputed SNPs dataset with 10,707,430 biallelic SNPs using an accelerated mixed model (AMM) (Seren et al., 2012). The ionomic data used was composed of the leaf concentrations of Na, Ca, Mg, B, P, K, Mn, Fe, Co, Cu, As, Zn, Cd and Mo. All significant SNPs (-log(*p*-value) > 5) and corresponding candidate genes were organized based on their *p*-values per phenotype and listed in Supplemental Data set 10. As was shown previously (Baxter et al., 2010; Chao

et al., 2012; Chao et al., 2014; Forsberg et al., 2015), the leaf concentrations of Na, Cd, As and Mo showed the strongest associations with causal genes that have been established as At-*HKT1;1* (AT4G10310), At-*HMA3* (AT4G30120), At-*HAC1* (AT2G21045), and At-*MOT1;1* (AT2G25680), respectively. We also newly found several strong SNPs which are highly associated with leaf Co or Zn concentrations (p-value < 10^{-9}) (Supplemental Data set 8). The most highly associated SNP for the variation in leaf Co concentration was at Chr5: 902186 (-log (p-value) = 29.08) within ~1 kb of the gene *IRON-REGULATED 2* (At-*IRGT2*/*FPN2*; AT5G03570). This gene has previously been shown to control leaf Co concentration using linkage mapping in a bi-parental F2 population derived from the accessions of Columbia-0 and Ts-1(collected in Tossa de Mar, Spain) (Morrissey et al., 2009). In addition, a number of SNPs with -log(p-value) > 5 for the leaf concentrations of Ca, Mg, B, P, K, Mn, Fe or Cu and candidate genes were also identified (Supplemental Data set 8).

We then extracted all genes within 300 kb of the lead SNPs in the rice GWAS data for each element to look for orthologous genes with candidates obtained from the *A. thaliana* GWAS data, using PLAZA Comparative Genomics Platform (PLAZA v2.5) (Van Bel et al., 2012; Tomcal et al., 2013). Only a total of 5 couples of orthologous genes were obtained for associations with As, Mn, Mo and Na (Supplemental Data set 9). The rice genes Os-*HKT1;5* and *Os-MOT1;1* associated with variation in rice shoot Na and Mo, respectively, were found to be paired with *At-HKT1;1* and *At-MOT1* previously determined to control the variation in these same elements in *A. thaliana* leaves. This provides strong evidence that the variations in Na and Mo are governed by the same genes across monocots and dicots. The comparative GWAS approach therefore also provides a useful and independent method to further validate the role of candidate genes identified using GWAS.

Rice ionome is affected by the heading date

Heading date is an important trait for the adaptation of rice plants to different growth environments (Jung and Muller, 2009). In general, rice accessions with later heading date

have a longer vegetative phase and consequently a larger yield potential. To test whether heading date influences the rice ionome, we analyzed the correlations between elemental concentrations and heading dates (Supplemental Figure 17). We found significant negative correlations between heading date and the concentrations of N, P, K, Ca, B, Cu, Fe and As, in some of the three plant tissues or field conditions at Wuhan. In contrast, there were significant positive correlations between heading date and Cd concentrations in all tissues from all three field conditions, as well as with Pb and Mn concentrations in some tissues and field conditions. The negative and positive correlations between heading date and As and Cd, respectively, were also observed in a panel of 467 locally adapted rice cultivars in south China (Duan et al., 2017).

Nitrogen is one of the most important nutrients limiting crop productivity, whereas overuse of N fertilizers can cause serious environmental damages. Increasing N use efficiency in crops is therefore an important goal to enhance agricultural sustainability. The locus defined by SNP sf0709172004 was found to be strongly associated with the concentrations of N in shoots at heading stage, whilst the nearby SNP sf0709177919 defined the locus for heading date (Figures 8A and 8B). Furthermore, the locus associated with N concentrations was still detected when heading date was used as a covariate (Figure 8C). We therefore selected this locus for further analysis. We found that the gene LOC_Os07g15770, known as *Hd4* or *Ghd7* and having a large effect on rice heading date, is located at this locus (~17 kb away from the lead SNP) (Xue et al., 2008). Because N concentration is strongly correlated with heading date (Supplemental Figure 17), we suggested that *Ghd7* is a likely candidate. According to the sequence data, there are 45 haplotypes of Ghd7 in all 529 accessions (Supplemental Data set 10). Within the *indica* accessions there are 5 main haplotypes (Hap1~Hap5) (Figures 8D, 8E and Supplemental Data set 10), among which Hap2 showed significantly shorter heading date but higher N concentrations than other haplotypes (Figure 8F and 8G). In fact, the nucleotide diversity of *Ghd7* genomic sequence had ever been analyzed in 104 rice

accessions and 58 of which were included in our collection (Lu et al., 2012; Supplemental Data set 10). Consistent with our findings, haplotypes of Ghd7 with longer heading dates showed relatively lower N concentrations in shoots. To investigate the effect of *Ghd7*, we compared N concentrations and heading dates among four near-isogenic lines (NIL) developed previously in the genetic background of Zhenshan 97 with the introgressed segment around the *Ghd7* region from different rice varieties (Xue et al., 2008). Three lines containing functional alleles of *Ghd7* [NIL(mh7), NIL(nip7) and NIL(tq7)] contained significantly lower N concentrations in various tissues than the line NIL(zs7) carrying a compete deletion in the corresponding region (Figures 9A to 9C). Furthermore, overexpressing a functional allele (mh7) of *Ghd7* with the ubiquitin promoter into Zhenshan 97 generally decreased N concentrations in shoots, straw and brown rice compared with wild-type (Figures 9D to 9F).

Loci involved in rice growth under low N or low P conditions

It has been suggested that N uptake explained most of the variation for nitrogen use efficiency (NUE) and grain yield at low N supply (Hirel et al., 2007). Variation in plant biomass production at low N or low P supply reflects the variation in the abilities to tolerate low N or low P, which are important traits for breeding rice cultivars that can produce acceptable levels of yield with reduced fertilizer inputs. In the present study, we found large variations among rice accessions in both the above-ground biomass and grain yield under low N or low P conditions (Supplemental Figure 18). We performed GWAS on above-ground biomass at both the heading and mature stages and grain yield. In our collection, biomass at both heading and mature stages correlated negatively with N concentrations but positively with the total amount of N in the plant (Supplemental Figure 19). Grain yield also correlated positively with total N of the plant, especially in the LN field. Loci that were associated with variation in biomass and grain yield at low N field are likely to be involved in controlling rice growth under low N fertilization. We

explored the loci identified from at least two traits (for instance, biomass at heading stage and grain yield) at low N field, low P field as well as normal field at Wuhan, respectively (Supplemental Table 7). Meanwhile, loci which were identified in the same tissue and subpopulation in at least two fields for each trait were also obtained. Based on the above criteria, we obtained 11 loci responsible for biomass and grain yield (Supplemental Table 7). Of these loci, two are co-located with either Ghd7 or DTH8/Ghd8, which are known to control the variation in biomass and grain yield (Xue et al., 2008; Wei et al., 2010; Yan et al., 2011). Interestingly, we found that 5 loci were exclusively detected in the LN or normal field whereas only 2 loci were common in both fields, suggesting different genetic basis for controlling biomass production under low N and normal field condition. In contrast, LP field shared all loci with normal or LN field. Furthermore, a locus defined by SNP sf1125356264 was found from all 3 field conditions, which may contribute to variations of biomass or grain yield under various N or P fertilization conditions.

Genetic basis of low N and low P affecting ionome

As described above, the concentrations of many elements were greatly affected by the N or P status (Supplemental Figure 1 and Supplemental Table 1). This may reflect the crosstalk between N or P and other elements and the adjustment of the ionome in response to low N or low P conditions. Moreover, as essential macronutrients, N or P deficiency may affect the transcription levels of many genes and alter root morphology (Hermans et al., 2006). It is likely that these effects are genotype dependent. Therefore, investigating the genetic basis of changing ionomic profile at low N or low P conditions may provide some clues to understanding the responses of ionome to the environment.

Based on the GWAS results of the rice ionome performed above, we extracted 32 loci associated with the concentrations of 11 elements (Supplemental Data set 11), which were scanned across at least two tissues in one field condition. It is interesting that the majority of these loci (19 of 32) were also common in two or more field conditions. In

contrast, 13 loci were specific to field conditions, including 7 for normal field, 5 for low N field and 1 for low P field, Identification of the causal genes underlying these loci would provide insights into how N and P impact the rice ionome, and would also benefit rice breeding that tailor the varieties to local conditions.

Discussion

As an essential component of all living systems, the natural ionomic variation has significance in evolution and adaptation (Lahner et al., 2003; Huang and Salt, 2016). Over the last decade, numerous genes and gene networks have been shown to be involved in controlling mineral nutrient and trace element homeostasis in plants. However, the loci that control natural ionomic variation are still largely unknown, especially in rice. By performing GWAS on the concentrations of 17 elements in different tissues of 529 rice accessions grown under different locations, years and field conditions, we have identified 72 locus-element associations with high reproducibility across plant tissues, growth stages and field conditions (Supplemental Data sets 2 to 5). Our study represents the most comprehensive GWAS of the rice ionome to date.

GWAS of the rice ionome has to overcome a number of limiting factors, including the relatively small variation in plant elemental concentrations because of the homeostasis of essential mineral nutrients and the usually large environmental variation. Consistent with previous reports on *A. thaliana* (Baxter et al., 2012) and rice (Pinson et al., 2015), we found that trace elements, especially the non-essential toxic elements, showed greater variations than macronutrients in rice tissues (Figure 1 and Supplemental Table 1), suggesting that the latter are more tightly regulated. It is possible that plants have evolved stronger mechanisms to control the internal fluctuation of essential nutrients, especially macronutrients (e.g. N, P, K), than for non-essential elements, because the concentrations of macronutrients need to be maintained within relatively narrow ranges that are optimal for growth and development. For non-essential elements

there would be little selection pressure to control their concentrations until they reach the levels of toxicity. Despite different ranges of variation, 13 of the 17 elements determined in our study were found to have a relatively high heritability within the same location (average $H^2 \geq 0.5$) (Supplemental Table 2 and 5), indicating a good genetic basis for their variations in agreement with previous studies on both *A. thaliana* and rice (Baxter et al., 2012; Pinson et al., 2015). In contrast, elements including B, Fe, Cr and Pb showed low heritability; the latter three elements are known to be prone to small soil or dust contamination inflating their concentrations in plant tissues. However, the heritability for most elements was lower when plants were grown at different locations (Supplemental Table 5), consistent with previous studies that genetic variation of elements was relatively strong across years at the same field location but relatively weak between field locations (Norton et al., 2012b). To overcome the limitations associated with genetic versus environmental variations, we conducted five field trials across different locations and years and analyzed ionomic profiles of different rice tissues of 529 rice accessions. This approach allowed us to identify loci that are reproducible across environments (Norton et al., 2012b; Norton et al., 2014). We obtained 32 loci (out of 72) that were common across field locations (Supplemental Data sets 2 to 4). Moreover, 37 loci are close to the QTLs associated with specific elemental concentrations in rice reported in previous studies using bi-parental crosses or association mapping (Supplemental Data set 5). These loci represent genetic variations that are stable across different environments and would be more useful for breeding purposes.

The high density of SNPs (~17 SNPs per kb in average) in our GWAS panel also facilitates high-resolution mapping. The resolution (within approximately 100 kb) (Si et al., 2016) is much higher than the loci generally defined with the interval QTL mapping approach (Supplemental Data set 5). Because of the slow LD decay in rice and relatively complex genetic architecture of elemental traits (Huang and Han, 2014), a locus in rice identified by GWAS typically covers more than 20 genes (Matsumoto et al., 2005).

Using the information of functional gene annotation or their orthologous genes in other plant species, plausible candidate genes for a number of loci identified by GWAS could be established (Supplemental Data set 7). We further provide strong evidence that Os-*HKT1;5* and Os-*MOT1;1* are the causal genes for the loci with the lead SNP sf0111478828 and sf0800123053, respectively, which cause variations in Na and Mo accumulation (Figures 4 to 7). Os-HKT1;5 is a known Na transporter involved in rice salt tolerance (Ren et al., 2005; Cotsaftis et al., 2012; Negrao et al., 2013; Platten et al., 2013). However, the relationship between Os-HKT1;5 haplotypes and Na accumulation remains unclear. For example, Cotsaftis et al. (2012) suggested L395V as an essential substitution affecting pore rigidity based on the 3D model of OsHKT1;5 protein, whereas Negrao et al. (2013) proposed P140A and R184H, but not L395V, as the causal alterations according to their associations with salt stress tolerance. Negrao et al. (2013) genotyped 392 rice accessions by EcoTILLING and presented 15 haplotypes of Os-HKT1;5, and then phenotypically characterized the most representative 59 accessions for association analysis. However, due to significant difference of Na accumulations between subpopulations (even between sub-subpopulations, e.g. IndI and IndII; Supplemental Figure 12), comparing the ability of different haplotypes (or SNPs) across subpopulations may not be appropriate. The strength of our study, compared with other previous studies, lies in the large number of rice accessions within each subspecies so that the effects of population structure can be excluded, and the reproducibility of the haplotype differences across different sites, years and conditions. Based on analysis of data from five field trials in each subpopulation, our results showed that neither L395V nor P140A had a consistent effect on Na concentration (Figure 5), suggesting that they probably have no or a weak function in Na variation. In contrast, we found that H184R is a key substitution separating strong and weak alleles of Os-*HKT1;5* and the weak alleles are present in almost all *japonica* and most *indica* accessions (Figures 4 and 5).

The second GWAS locus for which the causal gene was identified in our study is

Os-*MOT1;1*. This is the first gene identified in rice that controls Mo accumulation. Different from Os-HKT1;5 that affects Na accumulation via coding region variation, we found that the variation in Mo accumulation in rice is caused by the expression levels of Os-*MOT1;1* (Figures 6 and 7). This finding is similar to *MOT1;1* in *A. thaliana* (Baxter et al., 2008). Further efforts will be needed to identify the causal SNPs/INDELs resulting in expression level variation of Os-*MOT1;1* in promoter region.

In addition to Os-HKT1;5 and Os-MOT1;1, we have identified candidate genes for a number of loci controlling the concentrations of each of the 17 elements determined in rice (Supplemental Data set 7). For example, *OsNRAMP5*, encoding a major Mn transporter in rice (Sasaki et al., 2012; Yang et al., 2014), was scanned for Mn concentrations at both Wuhan and Youxian locations (Supplemental Figure 20). Similarly, *OsNRAMP5* has been identified by QTL mapping recently which contributes to Mn variation among rice accessions via variation in the expression level (Liu et al., 2017). Further investigations are needed to verify these candidate genes. Various approaches including expressing genes in yeast mutants, knocking-out and overexpressing candidates, or developing platform of gene expression variations by RNA sequencing can be used to pick and validate candidate genes in future work. It is also noteworthy that for most elements, repeated loci responsible for the same element are closely located on the same chromosome (Figure 3, Supplemental Data sets 2 to 4). These close loci should be considered together to aid validation of candidate genes.

Indica and *japonica* are two major subspecies in cultivated rice and have a large genetic differentiation, resulting in a variety of phenotypic differences (Huang et al., 2012b). In this study, we found that the large diversity was also reflected in the ionomic phenotypes with *japonica* accessions generally accumulating higher levels of most elements than *indica* accessions (opposite pattern for Cd; Supplemental Tables 3 and 6). Interestingly, we found that the GWAS loci for the same mineral element were mostly not co-localized in the subpopulations of *indica* and *japonica* (Supplemental Data sets 2

to 4), indicating different genetic components underlying the variations of ionomic traits between these two subspecies. This speculation is supported by the characteristics of the genes that control the variations of the rice ionome in our study. For example, the strong haplotype (or Hap2) of Os-HKT1;5 existed only in *indica* but was extremely rare in the *japonica* subpopulation. Os-MOT1;1 was specifically scanned in *indica* GWAS panels as a possible reason of much lower Mo concentration in *indica* accessions (Supplemental Figure 8). In both cases, *indica* subspecies harbors greater allelic and/or functional diversity than *japonica* subspecies. These subspecies differences suggest a great potential to breed across subpopulations for better nutrient uptake or salt tolerance.

For a number of agronomic traits including seed size, shattering habit and flowering time, and metabolic traits, locations of QTLs have been shown to be conserved across rice, sorghum and maize (Paterson et al., 1995; Chen et al., 2016), suggesting a convergent domestication underlying these phenotypes across cereals. Common causal or candidate genes were also found controlling the variations of Na (Os-*HKT1;5*/At-*HKT1*), Mo (Os-*MOT1;1*/At-*MOT1*) and Cd (Os-*HMA3*/At-*HMA3*) in rice and *A. thaliana* (Supplemental Data set 9) (Ueno et al., 2010; Chao et al., 2012), suggesting the conservation of genetic variation at specific loci across monocots and dicots. Moreover, Tm-HKT1;5-A in wheat (Munns et al., 2012), Zm-MOT1 (an ortholog of MOT1s) in maize (Asaro et al., 2016), and Tc-HMA3 in *Thlaspi caerulescens* (now *Noccaea caerulescens*) were also responsible for controlling variations of Na, Mo and Cd accumulation, respectively (Ueno et al., 2011). However, the functional SNPs/INDELs within each ortholog group were always different. For example, a 53 bp deletion 27 nucleotides upstream from the translation start site of At-MOT1 was suggested to be responsible for controlling the variation of expression and the shoot Mo concentration in *Arabidopsis* (Baxter et al., 2008), but this deletion was not found in the promoter region of Os-MOT1;1 in rice, suggesting that other polymorphisms control the expressional variation in rice (Supplemental Figure 15). Such difference suggests that the variations in

these loci were independently obtained by different plant species during their evolutionary selection, which cause similar ionomic consequences. It is unclear why selection was acting on the same genes across species, but such conservation provides a useful pathway to combine the discoveries of ionomic loci from different plant species and help mine candidate genes. It also means that the ionomic GWAS loci in rice identified in the present study can serve as a useful resource for other crop species.

Ghd7 is an important regulator of heading date, plant height and grain yield, and contributes to the variations of these traits in rice germplasm collections (Xue et al., 2008; Weng et al., 2014; Zhang et al., 2015). In this study, we have identified *Ghd7* as the causal gene for variations in the N concentrations in both shoots and straws (Figures 8 and 9). Interestingly, *Ghd7* has also been observed in a QTL related to N-deficiency tolerance in rice (Wei et al., 2012). Here we found that N concentration in rice collections was highly correlated with heading date (Supplemental Figure 17), and overexpressing a functional allele of *Ghd7* in rice plants significantly decreased N accumulation (Figure 8). A recent research showed that the enhanced expression of *Ghd7* lowered the chlorophyll content (Wang et al., 2015), which could be explained by the negative effect of Ghd7 on N concentration observed in our study. Another important gene for rice yield, *DEP1*, has recently been shown to regulate nitrogen-use efficiency in rice (Sun et al., 2014). Mutation of DEP1 resulted in decreased plant height and increased N concentration, but did not affect heading date. We suggest that these key genes of agronomic traits and nitrogen form a complex relationship in regulating plant growth and N accumulation. In our collection, we found significant negative correlation between biomass and N concentrations at both the heading and mature stages, but significant positive correlation between biomass and the total amount of N accumulated (Supplemental Figure 19). These opposite relationships suggest both that cultivars with higher biomass are able to acquire more N from the soil and that there is a dilution effect of increasing biomass on the N concentration. It is therefore important to identify loci that are associated together

with biomass, grain yield and N concentrations (Supplemental Table 7). These loci would provide more chances to investigate the relationship between N concentrations with biomass as well as grain yield, and also would benefit breeding rice with high nitrogen-use efficiency as well as high yield.

Reducing the inputs of N and P fertilizers while maintaining high yield and quality is crucial for agricultural sustainability. Crop breeding is an important tool for realizing this goal. The present study has shown that N and P deficiencies can affect the ionomic profile of rice plants, thus impacting grain quality by altering the concentrations of essential and toxic elements (Supplemental Figure 1 and Supplemental Table 1). Moreover, the extent of influence for different elements by nutritional status is obviously different. Analyses of the genetic basis of variation in the ionomic profile under different N and P supply conditions reveal both the conservative and non-conservative genetic components in controlling the ionomic profile in response to the altered nutrient status. Moreover, we have identified loci for element concentrations that are highly dependent on the N or P supply (Supplemental Data set 11). Isolation of the causal genes for these loci would help to unravel the complex crosstalk between N or P and other elements in rice plants.

In conclusion, we have described a comprehensive study of the rice ionome combining genetic methodologies with high-throughput elemental profiling to dissect the genetic basis of variations of 17 mineral elements in rice. Our study reveals at least 72 common locus-element associations showing high reproducibility under different field conditions and a large number of specific loci corresponding to different field conditions. We have identified strong candidates for three of the loci, including the first evidence that Os-MOT1;1 is involved in the variation of Mo accumulation and the effect of heading date gene *Ghd7* on N concentration in rice. The information of the locus-element associations obtained in the present study provides an important foundation for future studies on the genetic and molecular mechanisms controlling the rice ionome.

Methods

Plant Materials and Sequencing Data.

A set of 529 rice accessions was collected and sequenced as described previously (Chen et al., 2014). The set included 192 accessions from a core/minicore collection of *O. sativa* L. in China (Zhang et al., 2011), 132 parental lines used in the International Rice Molecular Breeding Program (Yu et al., 2003), 148 accessions from a minicore subset of the US Department of Agriculture rice gene bank (Agrama et al., 2009), 15 accessions used for SNP discovery in the OryzaSNP project (McNally et al., 2009), and 46 additional accessions from the Rice Germplasm Center at the IRRI. Information about the accessions, including names, countries of origin, geographical locations, and subpopulation classification, is listed in Supplemental Data set 1. We sequenced the 529 accessions using the Illumina HiSeq 2000 in the form of 90-bp paired–end reads to generate high-quality sequences of more than 1 Gb per accession (>2.5× per genome, total 6.7 billion reads). These raw data are available in NCBI with BioProject accession number PRJNA171289. The assembly release version 6.1 of genomic pseudomolecules of *japonica* cv. Nipponbare, downloaded from the rice annotation database of Michigan State University (MSU), was used as the reference genome. We performed imputation by using an in-house modified k nearest neighbour algorithm. The detailed procedures of data analysis have been described in our previous articles (Chen et al., 2014; Xie et al., 2015). The SNP information is available on the RiceVarMap website (Zhao et al., 2014).

The wild-type, mutant and transgenic plants related to Os-MOT1;1 used in the present study were all based on the background of the cv. Zhonghua 11 (O. *sativa* L. ssp. *japonica* variety). The *osmot1;1* mutant is a T-DNA insertion mutant (ID: 03Z11DK23) identified from the Rice Mutant Database (Wu et al., 2003; Zhang et al., 2006).

Field trials and Sample preparation

Field trials were conducted at two locations in China, including the experimental farm of Huazhong Agricultural University in Wuhan, Hubei province, and an experimental farm in Youxian, Hunan province. At Wuhan, the seeds of 529 rice accessions were germinated in a seed bed in mid May 2012 and were transplanted to three adjacent paddy fields with different nutritional status in mid-June 2012. The three different fields, including a normal field (NF) receiving standard fertilizer inputs, a low nitrogen field (LN) and a low phosphate field (LP), were constructed by controlling fertilizer applications in the recent decade. Fertilizers were applied (per hectare) as follows: 90 kg N, 45 kg P, and 72 kg K in the NF field; 45 kg P, and 72 kg K in the LN field; 90 kg N and 72 kg K in the LP field. At Youxian, the field trials were performed for two consecutive years (2014 and 2015). The seeds of 529 rice accessions were germinated in mid-May and the Seedlings about 25 days old were transplanted to the field in each year. Before rice transplanting, we randomly collected 11~13 soil samples in each field for the analyses of soil nutrient (and heavy metals/metalloids/pH values) status (Supplemental Tables 4). The irrigation water was also analyzed (Supplemental Tables 8). Each accession was planted in two replicates in each field at Wuhan and planted in three replicates for each year at Youxian, with 20 plants (two rows of ten plants) in each replicate, in a randomized complete block design. The planting density was 16.5 cm between plants in a row and 26 cm between rows. The trials were managed according to normal agricultural practices with regards to crop protection and paddy water management (considering the varied heading dates in different accessions, we drained the paddy water in early September for about 15 days, and then irrigated again for about a week, and drained the water in late September). Five plants of each accession were harvested at the heading stage, and another five plants were harvested at the maturity stage (40 days after flowering for each line). All plants taken for analysis were the ones in the inside lines. The five plants were combined to represent one biological replicate. Plant samples at maturity were separated into straw and brown rice (dehusked).

Elemental analysis

Plant samples were dried at 80 °C for 3 d and ground to fine powders. For the determinations of N and P concentrations, 0.2 g dried powder was digested with 5 ml of 98% H_2SO_4 and 5 ml of 30% hydrogen peroxide. After cooling, the digested sample was diluted to 100 ml with distilled water. The N concentration in the solution was determined colourimetrically at 660 nm using a modified Berthelot reaction with salicylate, dichloroisocyanurate and complex cyanides on an automated discrete analyser (SmartChem 200, France). The P concentration in the solution was measured using the molybdate blue method with absorbance read at 880 nm on an automated discrete analyser. For the determinations of other mineral elements, 0.2 g dried powder of each sample was digested in 65% nitric acid in a MARS6 microwave (CEM) with a gradient of temperatures from 120 °C to 180 °C for 45min. After dilution in deionized water, the concentrations of 15 elements determined by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7700 series, USA).

Genome-Wide Association Analyses

SNPs with minor allele frequency ≥ 0.05 and the number of rice accessions with the minor allele ≥ 15 in the population were used to carry out GWAS. In total, 2,767,191 and 1,857,866 SNPs were used in GWAS for subpopulations of *indica* and *japonica*, respectively. To control spurious associations, population structure was modeled as a random effect in LMM using the kinship (K) matrix. The evenly distributed random SNP set used to analyze population structure was used to calculate K. GWAS was performed by using LMM and LR provided by the FaST-LMM program. According to a modified Bonferroni correction described by Li et al. (Li et al., 2012), the effective number of independent SNPs (Me) in each population was calculated and then used to replace the total number of SNPs to determine the genome-wide significance thresholds of the

GWAS. The Me was calculated as 571,843 and 245,348 for the subpopulations of *indica* and *japonica*, respectively, and the suggestive *P* values were specified as 1.8×10^{-6} in *indica* and 4.1×10^{-6} in *japonica*. The corresponding thresholds were then set to identify significant association signals by LMM. To obtain independent association signals, multiple SNPs exceeding the threshold in a 5-Mb region were clustered by r^2 of LD ≥ 0.25, and SNPs with the lowest *P*-value in a cluster were considered as lead SNPs. The detailed method has been described in previous studies (Chen et al., 2014).

Vector construction and rice transformation

To generate the overexpression constructs of Os-*MOT1;1*, four types (Type 1 – 4) of full length cDNA of Os-*MOT1;1* were amplified by using primers Os-MOT1;1-OX-F/Os-MOT1;1-OX-R from four different rice accessions: W024, W102, C020 and C055, respectively. The amplified cDNA was first introduced into the gateway vector pDONR207 and then transferred into the destination vector pJC034 using the Gateway recombination reaction (Invitrogen). The constructs were transformed into cv. Zhonghua 11 by *Agrobacterium tumefaciens*-mediated transformation. To construct the *MOT1;1-promoter:GUS* plasmid, 2.5 kb of genomic sequence located upstream of the Os-*MOT1;1* initiation codon was amplified by using primers Os-MOT1;1-P-F/Os-MOT1;1-P-R from cv. Nipponbare genomic DNA. The amplified promoter fragment was then cloned into pDONR207 and then transformed into the Gateway-compatible GUS fusion vector pGWB3 by Gateway recombination reaction. Cv. Zhonghua 11 calli was transformed with this construct. The transgenic plant tissues were incubated in an X-Gluc staining buffer at 37 °C for 4 h. To generate the overexpression constructs of *Ghd7*, the full length cDNA of a functional allele of *Ghd7* was amplified by using primers Ghd7-OX-F/Ghd7-OX-R from Cv. Minghui 63. The amplified cDNA was first introduced into the pGEM-T vector (Promega) and then cloned into a modified overexpression vector PU1301^{GPF} with *Xho*1 and *Bam*H1 sites. All the primers used for

vector constructions were listed in Supplemental Table 9.

Hydroponic experiments

For expression analysis of Os-*MOT1;1* and the determination of Mo concentrations in roots and shoots of both transgenic and wild-type plants, hydroponic experiments were performed according the standard rice culture solution (1.44 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1.0 mM $CaCl_2$, 1.6 mM $MgSO_4$, 0.17 mM Na_2SiO_3, 50 μM Fe-EDTA, 0.06 μM $(NH_4)_6Mo_7O_{24}$, 15 μM H_3BO_3, 8 μM $MnCl_2$, 0.12 μM $CuSO_4$, 0.12 μM $ZnSO_4$, 29 μM $FeCl_3$ and 40.5 μM citric acid and adjust pH to 5.5 by sulfuric acid)(Yoshida et al., 1976). Rice plants were grown in full nutrient solution or Mo deficiency solution (full nutrient solution without Mo) for about four weeks after germination. The nutrient solution was renewed every 5 days. For both expressional and elemental analysis, three plants (or tissues from three different plants) were mixed in one replication, and three biological replicates were conducted for each line.

RNA extraction and real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen). The first-strand cDNAs were synthesized with 3 μg of total RNA in 20 μl of reaction mixture by using SuperScript III reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Real-time PCR was performed by using the SYBR Premix Ex TaqTM (TaKaRa) on an Applied Biosystems 7500 PCR instrument. The rice Ubiquitin5 gene was used as the internal control with primers qUbq-F/qUbq-R. The expression measurements were made by using the relative quantification method (Livak and Schmittgen, 2001). All the primers used for real-time PCR were listed in Supplemental Table 9.

Statistical analyses

Broad-sense heritability (H^2) was calculated using the following equation by treating

accessions as a random effect and the biological replication as a replication effect using one-way analysis of variance (ANOVA) as described previously (Chen et al., 2014): $H^2 = var_{(G)}/(var_{(G)} + var_{(E)})$, where var(G) and var(E) are the variance derived from genetic and environmental effects, respectively. LD was estimated by using standardized disequilibrium coefficients (D'), and squared allele-frequency correlations (r^2) for pairs of SNP loci were determined according to the TASSEL program (Bradbury et al., 2007). LD plots were generated in Haploview (Barrett, 2009), indicating r^2 values between pairs of SNPs multiplied by 100 and color coded with white ($r^2 = 0$), shades of gray ($0 < r^2 < 1$) and black ($r^2 = 1$).

Accession Numbers

Sequence data used in this article can be found in the GenBank/EMBL database under the following accession numbers: Os-HKT1;5 (Os01g0307500), Os-MOT1;1 (Os08g0101500), and Ghd7 (Os07g0261200).

Supplemental Data

Supplemental Figure 1. Box plots for elemental concentrations among different field conditions at Wuhan.

Supplemental Figure 2. Principal component analysis of ionome between normal and low fertilizer field conditions.

Supplemental Figure 3. Hierarchical clustering of natural accessions based on their ionome.

Supplemental Figure 4. Box plots for elemental concentrations at Wuhan field conditions.

Supplemental Figure 5. Box plots for elemental concentrations at Youxian field conditions.

Supplemental Figure 6. Box plots for concentrations of some elements at Wuhan field

conditions after the Box–Cox transformation.

Supplemental Figure 7. Box plots for concentrations of some elements at Youxian field conditions after the Box–Cox transformation.

Supplemental Figure 8. A presentation of some Manhattan and Q-Q plots which showed relatively well repeatability and significance for these elements.

Supplemental Figure 9. A presentation of local linkage disequilibrium for a few loci in corresponding diversity panels.

Supplemental Figure 10. A list for all haplotypes of Os-HKT1;5 in 1479 or 529 natural accessions.

Supplemental Figure 11. Geographical distribution of the accessions possessing different haplotypes of Os-HKT1;5 on part of the world map.

Supplemental Figure 12. Analysis of Na concentrations in different subgroups within each Os-HKT1;5 haplotype in our collection.

Supplemental Figure 13. Histochemical staining of GUS activity in rice plants transformed with the Os-*MOT1;1*-promoter:*GUS* construct.

Supplemental Figure 14. Identification of the T-DNA inserted line of Os-*MOT1;1*.

Supplemental Figure 15. Sequence alignment of Os-*MOT1;1* promoter region in *indica* subpopulation based on fully sequencing data of 20 *indica* accessions.

Supplemental Figure 16. Sequence alignment of Os-*MOT1;1* CDS in *indica* subpopulation based on fully sequencing data of 20 *indica* accessions.

Supplemental Figure 17 The correlation between heading date and iomome at Wuhan revealed by a heat map of correlation coefficient.

Supplemental Figure 18. Box plots for grain yield and biomass of natural population of cultivated rice at three different field conditions of Wuhan location.

Supplemental Figure 19. Correlation among N concentrations, biomass and grain yield in natural population of cultivated rice at Wuhan location.

Supplemental Figure 20. Characterization of Os-NRAMP5 by GWAS.

Supplemental Table 1. Means and standard deviations of elemental concentrations of natural population of cultivated rice in each tissue and at each filed of Wuhan location.

Supplemental Table 2. The broad-sense heritability (H^2) for each element across two biological replicates at Wuhan location.

Supplemental Table 3. The overall ratio of elemental concentrations between *indica* and *japonica* at location of Wuhan.

Supplemental Table 4. Soil nutrient status.

Supplemental Table 5. The broad-sense heritability (H^2) for each element across two consecutive years at Youxian location.

Supplemental Table 6. The overall ratio of elemental concentrations between *indica* and *japonica* at location of Youxian.

Supplemental Table 7. Co-localized GWAS loci among traits of above-ground biomass at heading stage, above-ground biomass at maturation stage and grain yield.

Supplemental Table 8. Elemental analysis in irrigation water.

Supplemental Table 9. Primers used in this study.

Supplemental Data set 1. Information of 529 accessions used in this study.

Supplemental Data set 2. The information of 41 common loci which were repeatedly scanned in at least two field conditions at Wuhan.

Supplemental Data set 3. The information of 14 common loci which were repeatedly scanned in two consecutive years at Youxian.

Supplemental Data set 4. The information of 32 common loci which were repeatedly scanned across locations of Wuhan and Youxian.

Supplemental Data set 5. Co-localization of the 72 common loci (from **Supplemental Data set 2, 3** and **4** after remove duplicates) with the previously detected element-related QTLs in rice.

Supplemental Data set 6. Gene list within 300 kb of the all 72 common loci which were repeatedly scanned at Wuhan or Youxian for 17 elements.

Supplemental Data set 7. Potential candidate genes (or known element-related genes) in the 72 loci which were repeatedly scanned for 17 elements.

Supplemental Data set 8. Ionomic GWAS data in *A. thaliana* based on leaf ionomic data of ~349 accessions.

Supplemental Data set 9. List of orthologous genes obtained by comparing ionomic GWAS data between rice and *A. thaliana*.

Supplemental Data set 10. The heading dates of 529 accessions used in this study when cultivated in Wuhan, China, and the Ghd7 haplotype for each accession based on its SNPs information.

Supplemental Data set 11. List of the loci which were scanned across tissues at Wuhan.

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AUTHOR CONTRIBUTIONS

X. L. conceived the project and supervised the study. M.Y., K.L., G.Y., Q.D., C.S., L.L., H.D., and J.H. performed the experiments. M.Y., K.L., F.J.Z., W.X., P.R., H.Z., X.Y.H. and L.M. analyzed the data. M.Y., W.X., H.Z., Z.Z., W.W., Y.X., G.W., J.X. and X.L. contributed reagents/materials/analysis tools. M.Y., F.J.Z. X.Y.H., D.E.S. and X.L. wrote the paper. All of the authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1. Hierarchical clustering on field conditions, subpopulations and tissues based on the ionome of a natural population of cultivated rice.

The clustering was performed using centralized concentrations of each element.

Figure 2. Presentation of a few well reproducible and significant Manhattan and Q-Q plots for these elements.

The first part of the title for each panel indicates the elemental types; the second part represents the location from which it was scanned; the third part shows its tissue types [specially at Wuhan: the first letter indicate field types ("C", "N" and "P" means "NF", "LN field" and "LP field" respectively); the second and third letter represent tissue types (HS: shoots at heading stage; MS: shoots at maturation stage; MG: brown rice)]; the last part represents subpopulation types (Ind: *indica*; Jap: *japonica*). Red arrows pointed to the loci repeatedly scanned in different field trials.

Figure 3. Distribution of 72 common loci which were repeatedly scanned within or across locations on 12 chromosomes according to physical distance.

The number on the left side of the column represents the physical location (Mb) of each lead SNP. The letters to the right of the column represent the corresponding elements. Different color referred to different location from which the locus was scanned. Blue, Wuhan; green, Youxian; red, both locations. Underline indicated the locus scanned at Wuhan by all three fields or at Youxian by all two fields. One and two asterisks implied the locus was not only scanned across location but also repeatedly scanned within one and two locations respectively. The Venn diagram displayed the numbers of repeated loci within or across locations.

Figure 4. GWAS for Na concentration and identification of the candidate gene Os-*HKT1;5* for the repeatedly scanned locus on chromosome 1.

(**A**) and (**B**) Manhattan and Q-Q plots for straw Na concentrations in *indica* subpopulation at locations of Wuhan (A; LP field) and Youxian (B; 2015), respectively. Red arrows pointed to the lead SNPs which were located close to Os-*HKT1;5*.

(**C**) Gene model of Os-*HKT1;5*. Filled black boxes represented coding sequence. The gray vertical lines marked the polymorphic sites identified by high-throughput sequencing, and the star represented the potentially functional site.

(**D**) A representation of pairwise r^2 values (a measure of LD) among all polymorphic sites in Os-*HKT1;5*, where the darkness of the color of each box corresponded to the r^2 value according to the legend.

(**E**) A list for 9 SNPs which leaded to 9 amino-acid changes in coding sequence of Os-HKT1;5. Red contents indicated the four variations previously identified. The two asterisks indicated the SNP showing strong linkage with lead SNP of this locus.

(**F**) to (**J**) Box plot for Na concentrations in straws at SNP sf0111461701. **F**, normal field at Wuhan; **G**, LN field at Wuhan; **H**, LP field at Wuhan; **I**, 2014 at Youxian; **J**, 2015 at Youxian.

Figure 5. Analysis of Na concentrations in different Os-HKT1;5 haplotypes in our collection.

Significant differences at $P < 0.05$ within each group are indicated by different letters (one way ANOVA test).

Figure 6. Characterization of Os-MOT1;1 by GWAS.

(**A**) and (**B**) Manhattan and Q-Q plots displaying the GWAS result of Mo concentrations of straw in *indica* subpopulation at Wuhan (LP field; **A**) and Youxian (2014; **B**)

respectively.

(**C**) Gene model of Os-*MOT1;1*. Filled black boxes represented the coding sequence. The gray vertical lines marked the polymorphic sites identified by high-throughput sequencing, and the stars represented the potentially functional site.

(**D**) A representation of pairwise r^2 values (a measure of LD) among all polymorphic sites in Os-*MOT1;1*, where the darkness of the color of each box corresponded to the r^2 value according to the legend.

(**E**) to (**I**) Box plot for Mo concentrations of *indica* subpopulation in different field conditions at SNP sf0800008059.

(**J**) Detecting the difference of Os-*MOT1;1* promoter region by PCR. Blue and red horizontal line indicated fragments from T type and C type of *indica* accessions at SNP sf0800008059, respectively.

(**K**) Schematic presentation of the genomic structure and sequence variations of Os-*MOT1;1* in T type and C type of *indica* accessions at SNP sf0800008059. The Os-*MOT1;1* sequence of Nipponbare downloaded from rice annotation database of Michigan State University (MSU) was used as the reference sequence. The Os-*MOT1;1* sequences of T type and C type of *indica* accessions at SNP sf0800008059 were based on a fully sequenced data of 10 accessions in each type. The black filled boxes in the promoter and CDS regions, open triangles and short vertical lines indicated sequence deletions, insertions and single base mutations, respectively.

(**L**) and (**M**) Os-*MOT1;1* expression at roots of different types of *indica* accessions under Mo sufficient condition (**L**) and Mo deficient condition (**M**). Data are shown as the means of three biological replicates ± SD, and roots of three plants were mixed in one replication. The *P* value is calculated using the *t* approximation.

Figure 7. Functional analysis of Os-MOT1;1 based on transgenic plants.

(**A**) Schematic presentation of four kinds of Os-*MOT1;1* CDS used for constructing

over-expressed rice plants. The vertical thick lines represented sequence deletions or single base mutations.

(**B**) The expression levels of Os-*MOT1;1* in wild-type (Zhonghua 11) and transgenic lines by qRT-PCR. Six transgenic lines of each type Os-MOT1;1 were analyzed, and among these, three lines possessed relative low expression levels and the other three possessed relative high expression levels. Data are shown as the means of three biological replicates ± SD, and leaf blade from three plants were mixed in one replication.

(**C**) and (**D**) The determination of Mo concentrations in roots (**C**) and shoots (**D**) of wild-type and transgenic plants. Data are shown as the means of three biological replicates ± SD, and roots or shoots of three plants were mixed in one replication. Significant differences at $P < 0.05$ among different groups are indicated by different letters (one way ANOVA test).

(**E**) and (**F**) Scatter plot for Mo concentrations and expression levels of Os-*MOT1;1* in roots (**E**) and shoots (**F**) of wild-type and transgenic plants.

Figure 8. Characterization of *Ghd7* in rice N accumulation by GWAS.

(**A**) Manhattan and Q-Q plots displaying the GWAS results of N concentrations in shoots of *indica* subpopulation at heading stage at LP field in Wuhan.

(**B**) Manhattan and Q-Q plots displaying the GWAS results of heading dates of *indica* subpopulation.

(**C**) Manhattan and Q-Q plots displaying the GWAS results of N concentrations in shoots of *indica* subpopulation at heading stage at LP field in Wuhan by using heading dates as a covariate. Red arrows in **A, B** and **C** pointed to a same lead SNP which was located close to *Ghd7*.

(**D**) Gene model of *Ghd7*. The black filled boxes represented coding sequence. The gray vertical lines marked the polymorphic sites identified by high-throughput sequencing in

indica subspecies.

(**E**) Haplotype analysis of *Ghd7* gene region in *indica* subspecies based on polymorphic sites shown in **D**. Only haplotypes with total number of accessions ≥ 5 were analyzed.

(**F**) Box plot for heading dates of different Ghd7 haplotypes.

(**G**) to (**I**) Box plots for shoot N concentrations of different Ghd7 haplotypes at heading stage in normal field (**G**), LN field (**H**) and LP field (**I**) in Wuhan. Significant differences at $P < 0.05$ within each group are indicated by different letters (one way ANOVA test).

Figure 9. Functional identification of *Ghd7* in rice N accumulation based on near-isogenic lines and transgenic plants.

(**A**) to (**C**) N concentrations in shoots at heading stage (**A**), in straw (**B**) and in brown rice (**C**), of four near-isogenic lines.

(**D**) to (**F**) N concentrations in shoots at heading stage (**D**), in straw (**E**) and in brown rice (**F**), of wild type and transgenic plants.

Data are shown as the means of three biological replicates ± SD, and corresponding tissues of five plants were mixed in one replication for metal determination. Two asterisks indicate significant differences (P <0.01) compared with the wild type (*t* test).

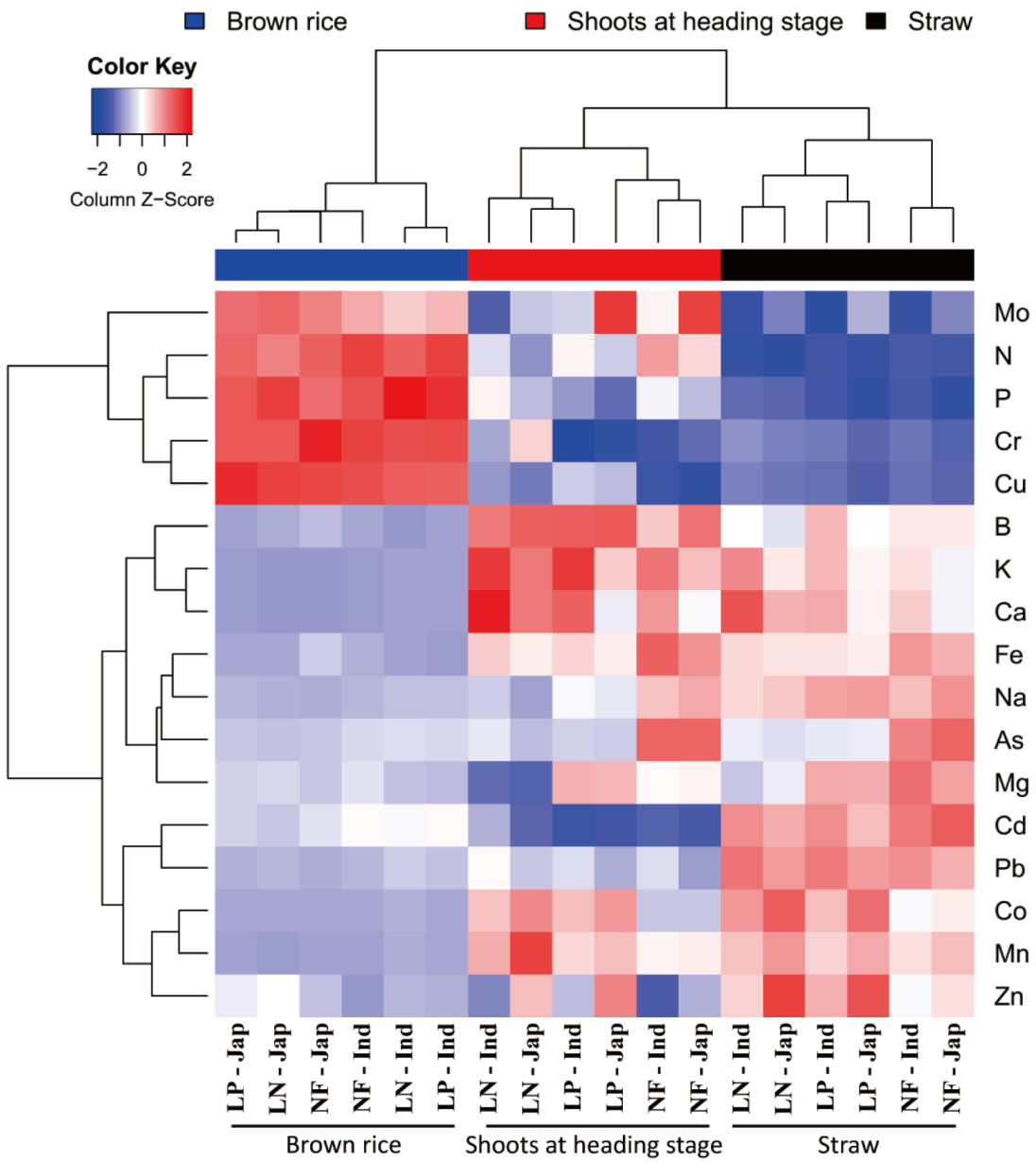


Figure 1. Hierarchical clustering on field conditions, subpopulations and tissues based on the ionome of a natural population of cultivated rice.
The clustering was performed using centralized concentrations of each element.

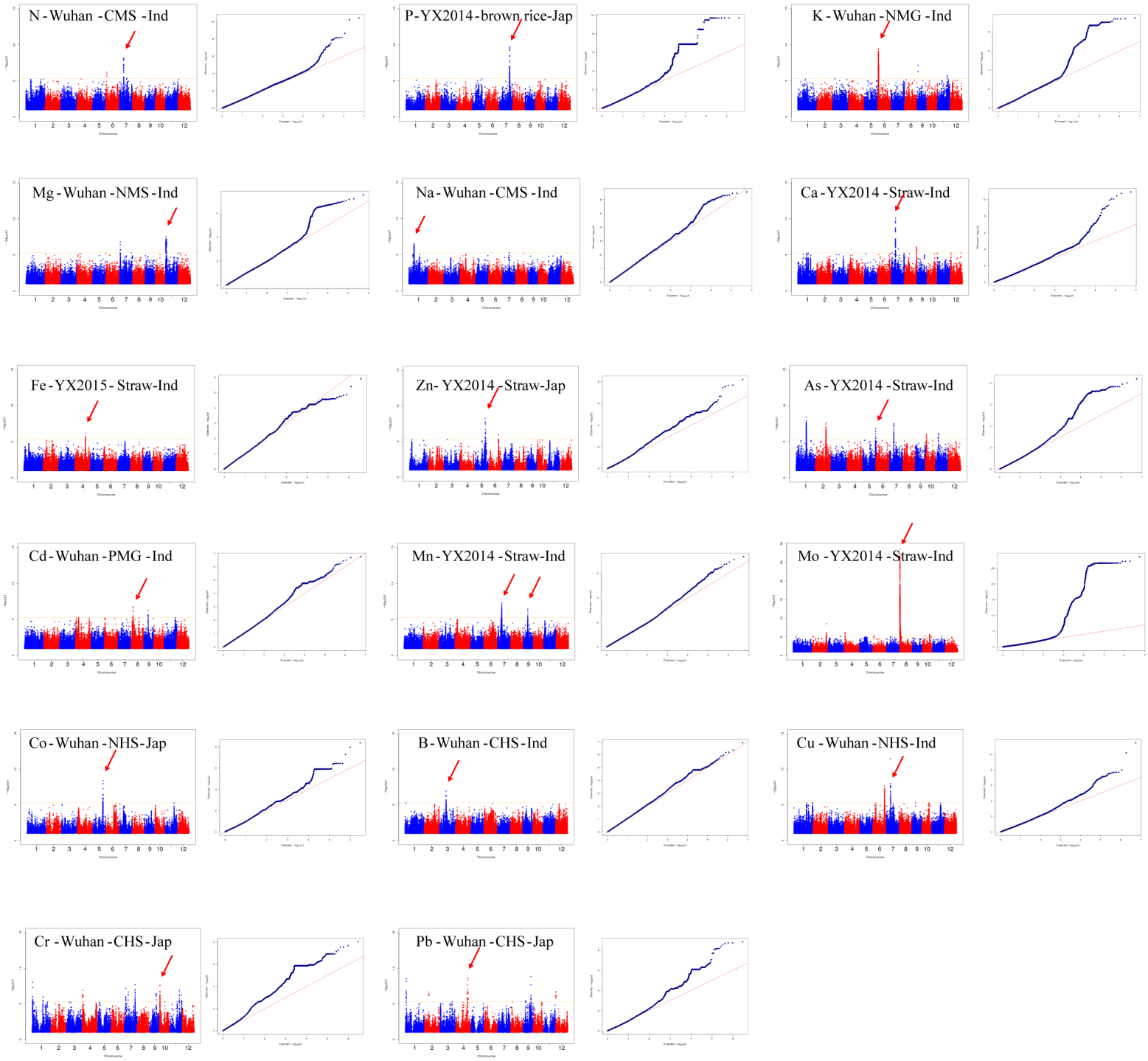


Figure 2. Presentation of a few well reproducible and significant Manhattan and Q-Q plots for these elements. The first part of the title for each panel indicates the elemental types; the second part represents the location from which it was scanned; the third part shows its tissue types [specially in Wuhan: the first letter indicate field types ("C", "N" and "P" means "NF", "LN field" and "LP field" respectively); the second and third letter represent tissue types (HS: shoots at heading stage; MS: shoots at maturation stage; MG: brown rice)]; the last part represents subpopulation types (Ind: *indica*; Jap: *japonica*). Red arrows pointed to the loci repeatedly scanned in different field trials.

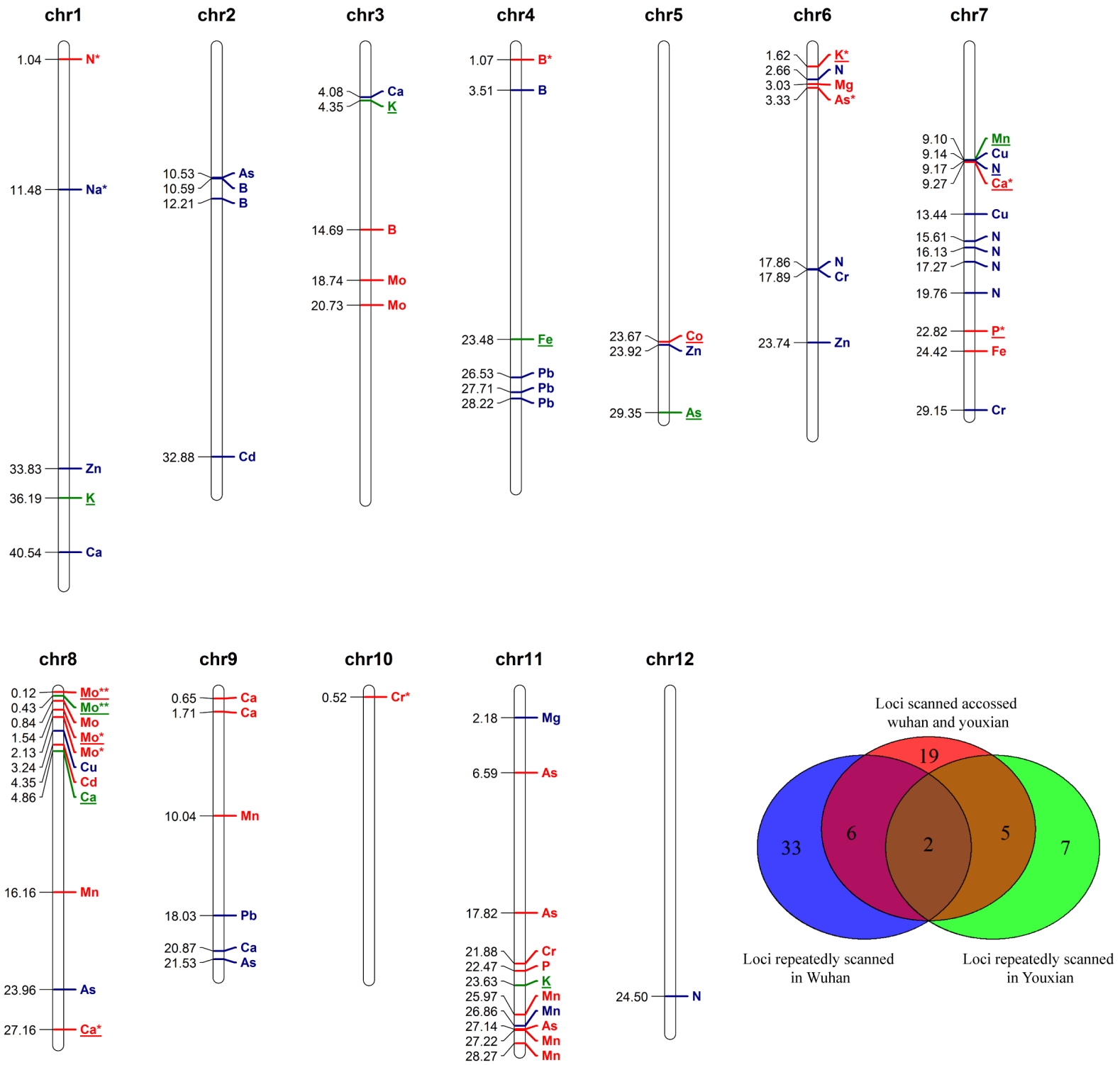
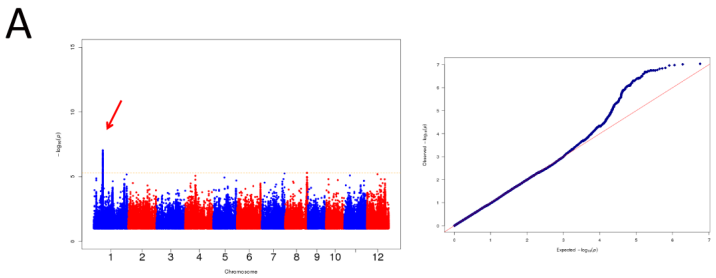
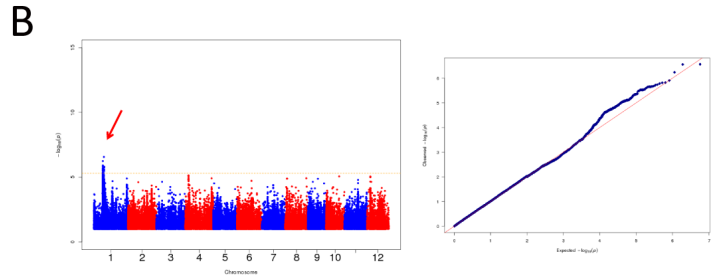
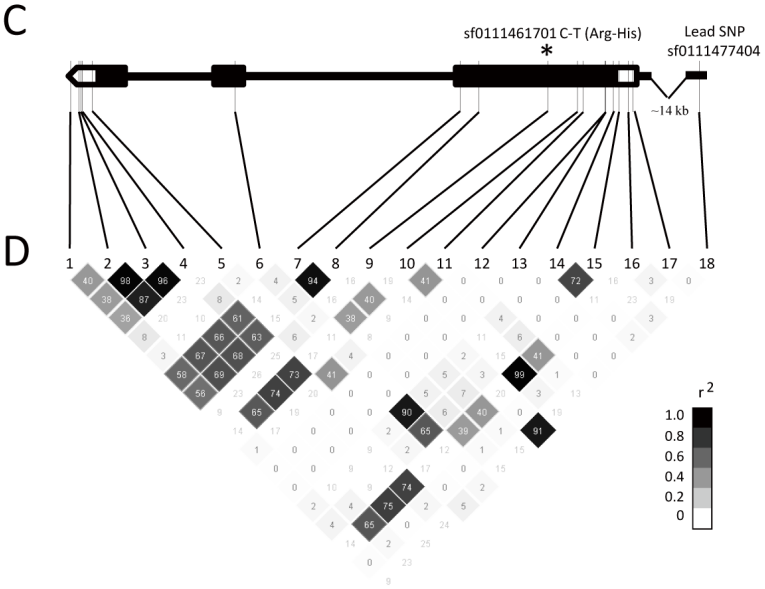


Figure 3. Figure 3. Distribution of 72 common loci which were repeatedly scanned within or across locations on 12 chromosomes according to physical distance.

The number on the left side of the column represents the physical location (Mb) of each lead SNP. The letters to the right of the column represent the corresponding elements. Different color referred to different location from which the locus was scanned. Blue, Wuhan; green, Youxian; red, both locations. Underline indicated the locus scanned in Wuhan by all three fields or in Youxian by all two fields. One and two asterisks implied the locus was not only scanned across location but also repeatedly scanned within one and two locations respectively. The Venn diagram displayed the numbers of repeated loci within or across locations.







E

Serial Number	SNP ID	Reference/ SNP allele	Reference/ AA allele	Numbers in all population	Numbers in indica	Numbers in japonica
14	sf0111462247	C/T	AA^{2th} S/N	487/42	275/20	156/0
13	sf0111462054	G/C	AA^{66th} F/L	528/1	295/0	155/1
12	sf0111462052	G/T	AA^{67th} T/K	527/1	295/0	154/1
11	sf0111461870	C/T	AA^{128th} D/N	338/191	115/180	153/3
10	sf0111461834	G/C	AA^{140th} P/A	223/306	38/257	152/2
9**	sf0111461701	C/T	AA^{184th} R/H	414/115	218/77	155/1
8	sf0111461258	G/C	AA^{332th} H/D	199/330	35/260	151/5
7	sf0111461069	G/C	AA^{395th} L/V	196/333	32/263	147/9
6	sf0111459320	G/T	AA^{429th} Q/K	483/46	274/21	154/2

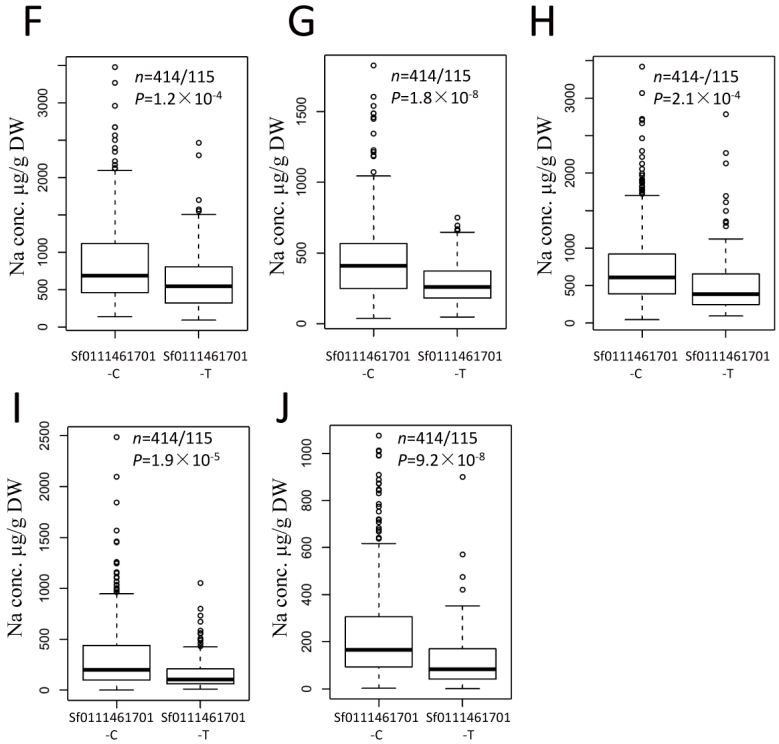


Figure 4. GWAS for Na concentration and identification of the candidate gene Os-*HKT1;5* for the repeatedly scanned locus on chromosome 1.

(**A**) and (**B**) Manhattan and Q-Q plots for straw Na concentrations in *indica* subpopulation at locations of Wuhan (**A**; LP field) and Youxian (**B**; 2015), respectively. Red arrows pointed to the lead SNPs which were located close to Os-*HKT1;5*.

(**C**) Gene model of Os-*HKT1;5*. Filled black boxes represented coding sequence. The gray vertical lines marked the polymorphic sites identified by high-throughput sequencing, and the star represented the potentially functional site.

(**D**) A representation of pairwise r^2 values (a measure of LD) among all polymorphic sites in Os-*HKT1;5*, where the darkness of the color of each box corresponded to the r^2 value according to the legend.

(**E**) A list for 9 SNPs which leaded to 9 amino-acid changes in coding sequence of Os-HKT1;5. Red contents indicated the four variations previously identified. The two asterisks indicated the SNP showing strong linkage with lead SNP of this locus.

(**F**) to (**J**) Box plot for Na concentrations in straws at SNP sf0111461701. **F**, normal field at Wuhan; **G**, LN field at Wuhan; **H**, LP field at Wuhan; **I**, Youxian at 2014; **J**, Youxian at 2015.

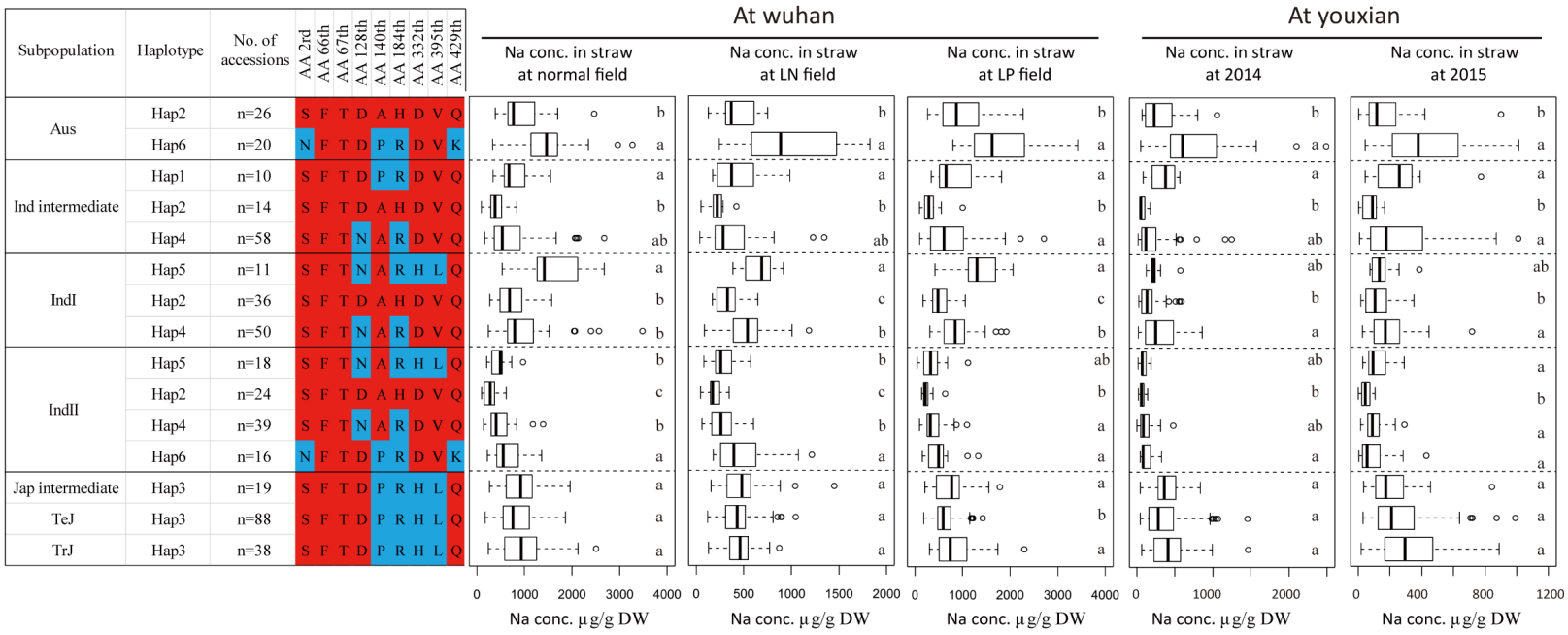


Figure 5. Analysis of Na concentrations in different Os-HKT1;5 haplotypes in our collection. Significant differences at $P < 0.05$ within each group are indicated by different letters (one way ANOVA test).

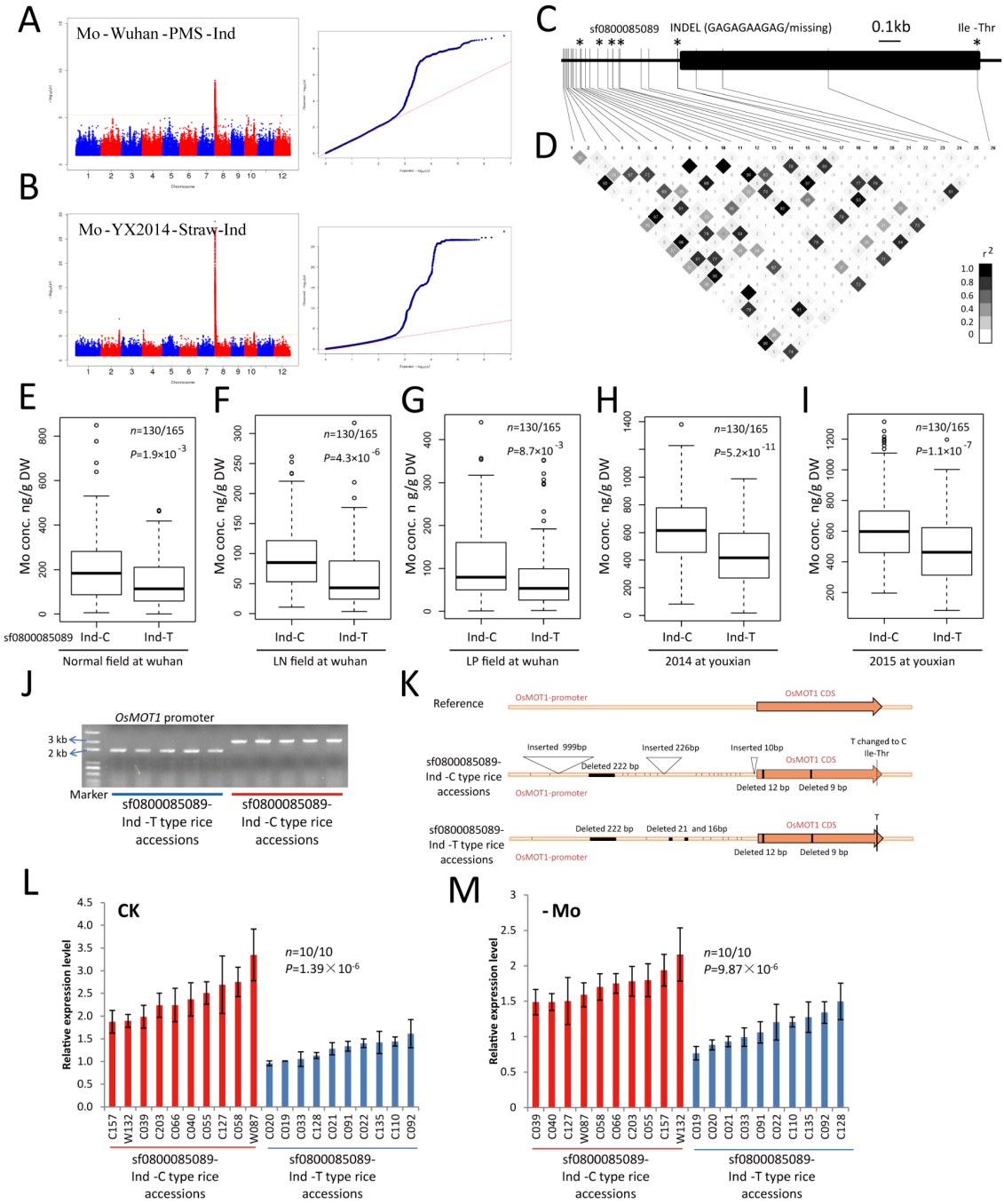


Figure 6. Characterization of Os-MOT1;1 by GWAS.

(**A**) and (**B**) Manhattan and Q-Q plots displaying the GWAS result of Mo concentrations of straw in *indica* subpopulation at Wuhan (LP field; **A**) and Youxian (2014; **B**) respectively.

(**C**) Gene model of Os-*MOT1;1*. Filled black boxes represented the coding sequence. The gray vertical lines marked the polymorphic sites identified by high-throughput sequencing, and the stars represented the potentially functional site.

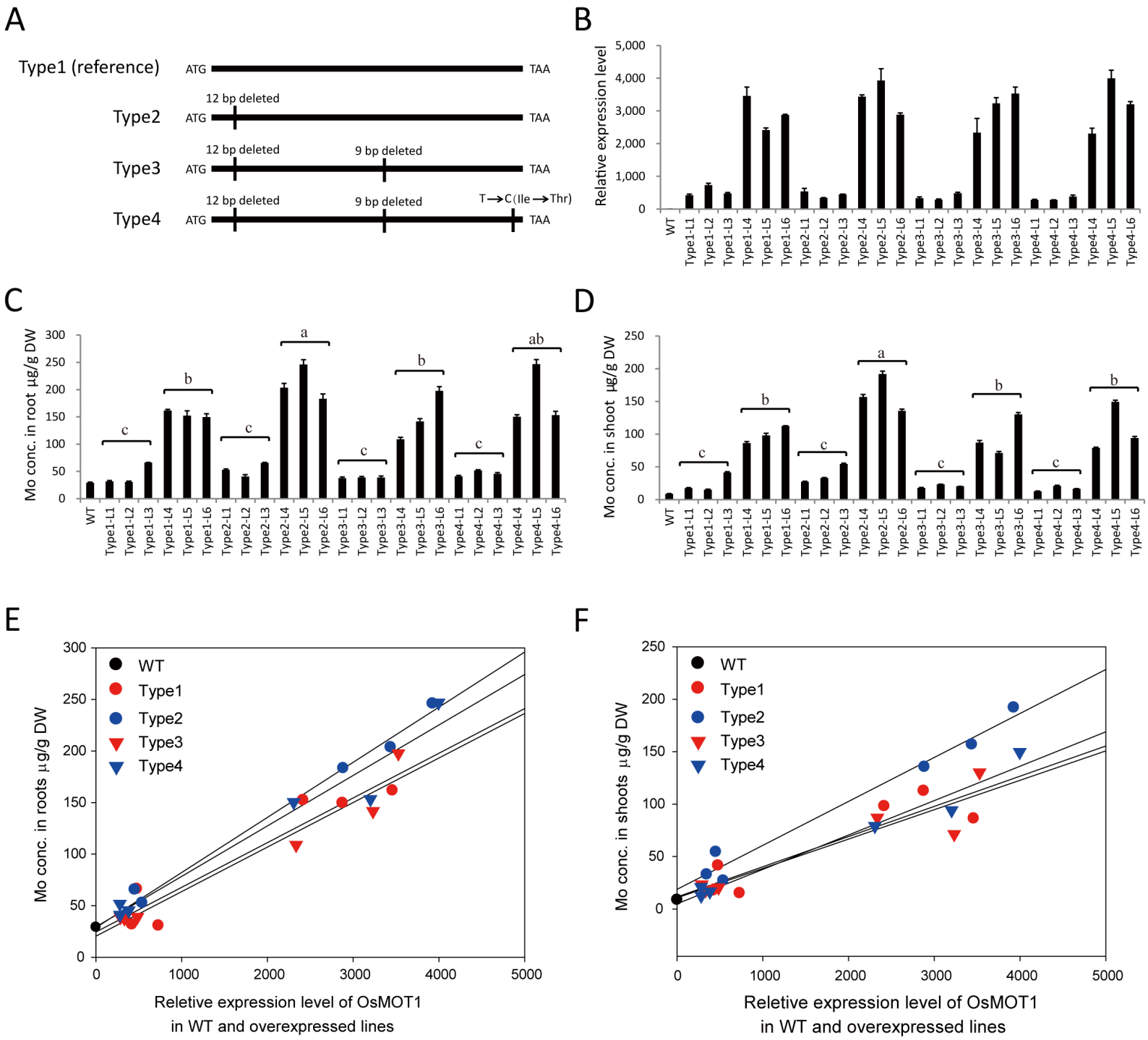
(**D**) A representation of pairwise r^2 values (a measure of LD) among all polymorphic sites in Os-*MOT1;1*, where the darkness of the color of each box corresponded to the r^2 value according to the legend.

(**E**) to (**I**) Box plot for Mo concentrations of indica subpopulation in different field conditions at SNP sf0800008059.

(**J**) Detecting the difference of Os-*MOT1;1* promoter region by PCR. Blue and red horizontal line indicated fragments from T type and C type of *indica* accessions at SNP sf0800008059, respectively.

(**K**) Schematic presentation of the genomic structure and sequence variations of Os-*MOT1;1* in T type and C type of *indica* accessions at SNP sf0800008059. The Os-*MOT1;1* sequence of Nipponbare downloaded from rice annotation database of Michigan State University (MSU) was used as the reference sequence. The Os-*MOT1;1* sequences of T type and C type of *indica* accessions at SNP sf0800008059 were based on a fully sequenced data of 10 accessions in each type. The black filled boxes in the promoter and CDS regions, open triangles and short vertical lines indicated sequence deletions, insertions and single base mutations, respectively.

(**L**) and (**M**) Os-*MOT1;1* expression at roots of different types of *indica* accessions under Mo sufficient condition (**L**) and Mo deficient condition (**M**). Data are shown as the means of three biological replicates ± SD, and roots of three plants were mixed in one replication. The *P* value is calculated using the *t* approximation.



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(**A**) Schematic presentation of four kinds of Os-*MOT1;1* CDS used for constructing over-expressed rice plants. The vertical thick lines represented sequence deletions or single base mutations.
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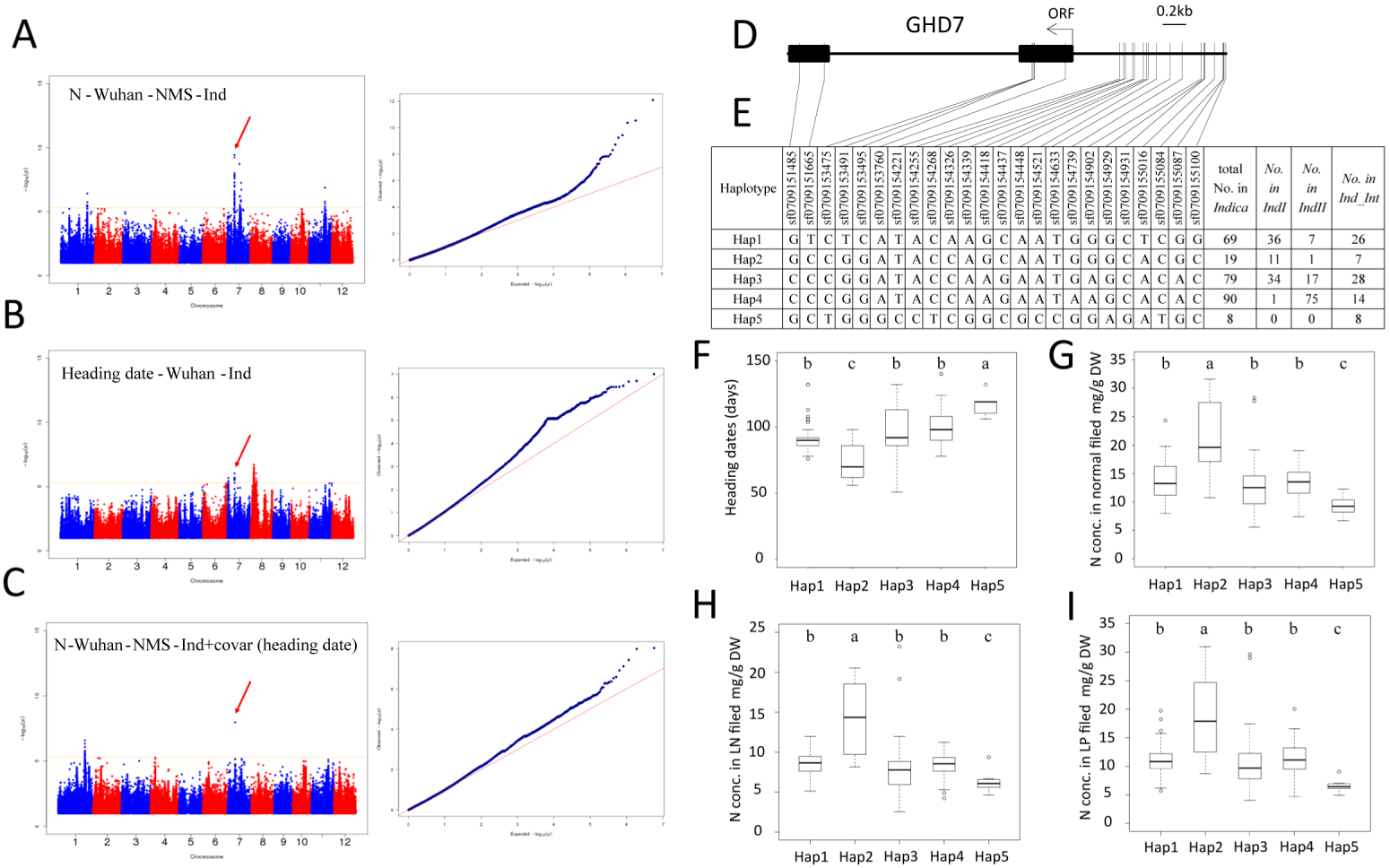
(**E**) and (**F**) Scatter plot for Mo concentrations and expression levels of Os-*MOT1;1* in roots (**E**) and shoots (**F**) of wild-type and transgenic plants.



E

Haplotype	s0709151485	s0709151665	s0709153475	s0709153491	s0709153495	s0709153760	s0709154221	s0709154255	s0709154268	s0709154326	s0709154339	s0709154418	s0709154437	s0709154448	s0709154521	s0709154633	s0709154739	s0709154902	s0709154929	s0709154931	s0709155016	s0709155084	s0709155087	s0709155100	total No. in *Indica*	*No. in IndI*	*No. in IndII*	*No. in Ind_Int*
Hap1	G	T	C	T	C	A	T	A	C	A	A	G	C	A	A	T	G	G	G	C	T	C	G	G	69	36	7	26
Hap2	G	C	C	G	G	A	T	A	C	C	A	G	C	A	A	T	G	G	G	C	A	C	G	C	19	11	1	7
Hap3	C	C	C	G	G	A	T	A	C	C	A	A	G	A	A	T	G	A	G	C	A	C	A	C	79	34	17	28
Hap4	C	C	C	G	G	A	T	A	C	C	A	A	G	A	A	T	A	A	G	C	A	C	A	C	90	1	75	14
Hap5	G	C	T	G	G	G	C	C	T	C	G	G	C	G	C	C	G	G	A	G	A	T	G	C	8	0	0	8

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(**A**) Manhattan and Q-Q plots displaying the GWAS results of N concentrations in shoots of *indica* subpopulation at heading stage at LP field in Wuhan.

(**B**) Manhattan and Q-Q plots displaying the GWAS results of heading dates of *indica* subpopulation.

(**C**) Manhattan and Q-Q plots displaying the GWAS results of N concentrations in shoots of *indica* subpopulation at heading stage at LP field in Wuhan by using heading dates as a covariate. Red arrows in **A**, **B** and **C** pointed to the lead SNPs which were located close to Ghd7.

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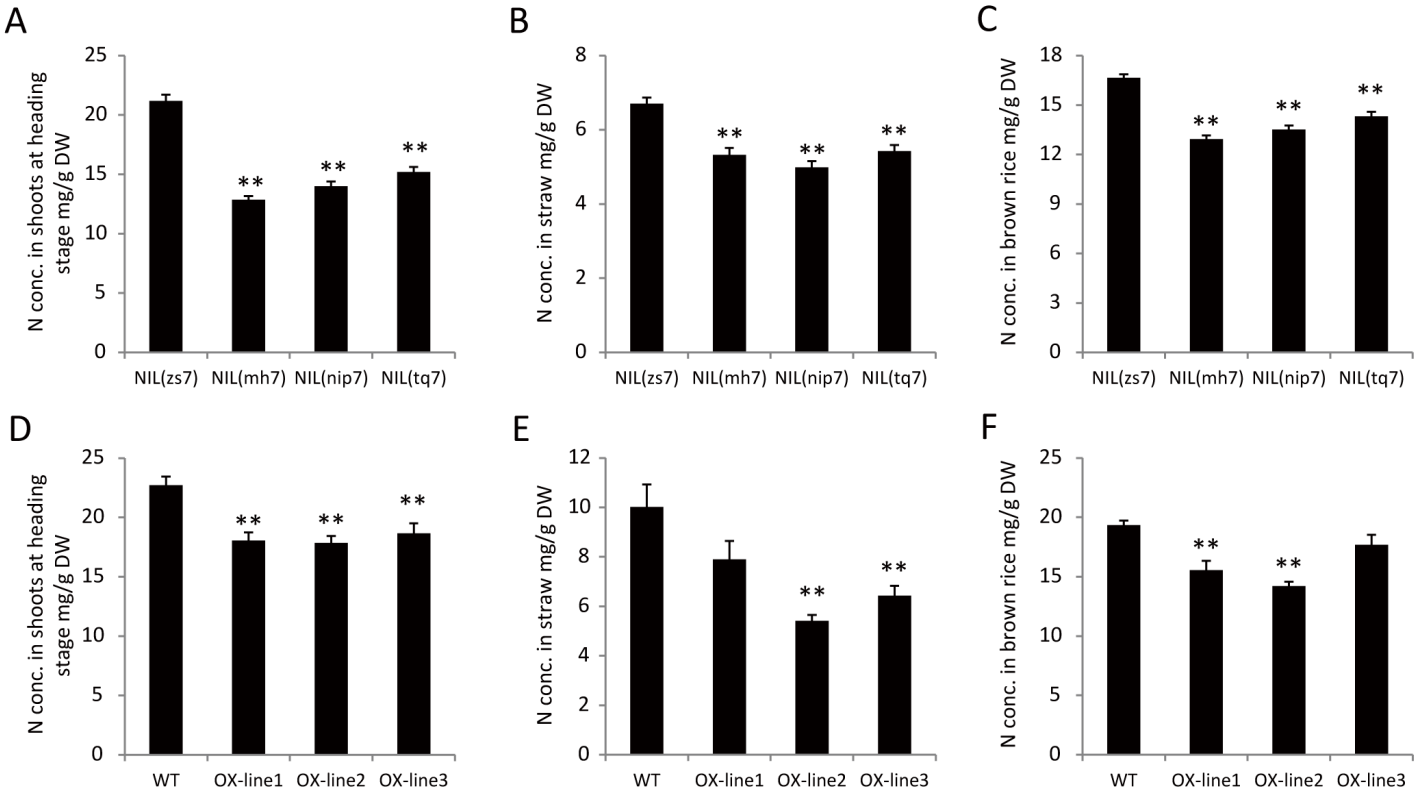
(**E**) Haplotype analysis of *Ghd7* gene region in *indica* subspecies based on polymorphic sites shown in **D**. Only haplotypes with total number of accessions ≥ 5 were analyzed.

(**F**) Box plot for heading dates of different Ghd7 haplotypes.

(**G**) to (**I**) Box plots for shoot N concentrations of different Ghd7 haplotypes at heading stage in normal field (**G**), LN field (**H**) and LP field (**I**) in Wuhan. Significant differences at $P < 0.05$ within each group are indicated by different letters (one way ANOVA test).



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(**A**) to (**C**) N concentrations in shoots at heading stage (**A**), in straw (**B**) and in brown rice (**C**), of four near-isogenic lines.

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Data are shown as the means of three biological replicates ± SD, and corresponding tissues of five plants were mixed in one replication for metal determination. Two asterisks indicate significant differences ($P < 0.01$) compared with the wild type (*t* test).

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