

Genome-Wide Association Study of Rice Rooting Ability at the Seedling Stage

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Abstract

Background Rice rooting ability is a complex agronomical trait that displays heterosis and plays an important role in rice growth and production. Only a few quantitative trait loci (QTLs) have been identified by bi-parental population. More genes or QTLs are required to dissect the genetic architecture of rice rooting ability.

Results To characterize the genetic basis for rice rooting ability, we used a natural rice population, genotyped by a 90K single nucleotide polymorphism (SNP) array, to identify the loci associated with rooting-related traits through the genome-wide association study (GWAS). Population structure analysis divided the natural population into two subgroups: *indica* and *japonica*. We measured four traits for evaluating rice rooting ability, namely root growth ability (RGA), maximum root length (MRL), root length (RL), and root number (RN). Combined with the association study in three panels consisting of one for the full population, one for *indica*, and one for *japonica*, 32 SNPs associated with rooting ability-related traits were identified. Through comparison of the relative expression levels and DNA sequences between germplasms with extreme phenotypes, results showed that *LOC_Os05g11810* had non-synonymous variations at the coding region, which may cause differences in root number, and that the expression levels of *LOC_Os04g09900* and *LOC_Os04g10060* are closely associated with root length variation.

Conclusions The goal of our research was to improve understanding of the genetic basis of rice rooting ability and provide useful molecular markers and germplasms for rice root breeding.

Background

Rice is the staple food for most regions in Asia and feeds more than half of the world's population (Hoang et al. 2019; Jiang et al. 2019). Rice production faces challenges posed by decreasing cultivated land and water resources. The root system of rice plays a key role in absorbing nutrients and water. Rice root is also an important synthetic organ of some metabolites and closely related to above-ground agronomical traits (Liang et al. 2011). Rice with a vigorous root system has greater tolerance to climatic factors (Mahender et al. 2015) and is better equipped to compete with weeds (Anandan et al. 2016).

Most research on rice root has concentrated on investigating seedling root morphological traits and physiological and biochemical indexes under different stress conditions (Rohila et al. 2019). We classified the cloned root-related genes into two categories based on function. The first category included the genes that regulate root system morphological characters, such as *ARL1/CRL1*, *SRT5/ Oscyt-inv1*, *OsGNA1*, *WOX11*, and *ARL1/CRL1* (Inukai et al. 2005; Liu et al. 2005). *ARL1* encodes a protein with a LATERAL ORGAN BUONDARIES (LOB) domain, which is involved in auxin-mediated cell dedifferentiation and controlled the initiation of adventitious root primordia in rice. *SRT5/ Oscyt-inv1* encodes a putative cytosolic Inv-N that cleaves sucrose at pH7.0 ~ pH8.0 and is the key isoform of Inv-Ns required for carbon and energy supply during early root development (Yao et al. 2009). The *Oscyt-inv1* mutant had an accumulation of sucrose but reduction of hexose. The cell length along the longitudinal axis of the root

was reduced, and the cell shape in the root elongation zone shrank (Jia et al. 2008). *OsGNA1* encodes a Glucosamine-6-Phosphate acetyltransferase that is involved in de novo UDP-N-acetylglucosamine biosynthesis and influences the cell metabolism, microtubule stabilization, and cell shape in rice roots (Jiang et al. 2005). *WOX11*, a WUSCHEL-related homeobox gene, is involved in the activation of the crown root emergence and growth. It can interact with the AP2/ERF protein, *ERF3*, which is involved in auxin- and cytokinin-responsive gene expression. The two genes function cooperatively to influence the crown root development by regulating the gene expression involved in cytokinin signaling (Chen et al. 2015; Zhao et al. 2009; Zhao et al. 2015). The second category of cloned genes was genes related to abiotic stress tolerance, like *OsP5CS* and *OsNAC9*. *OsP5CS* encodes a Δ^1 -pyrroline-5-carboxylate synthetase and plays an important role in proline accumulation under salt stress. Overexpression *OsP5CS* transgenic plants showed a better root growth (Zhu et al. 1998). *OsNAC9* encodes a novel NAC-domain transcription factor and overexpressing transgenic lines transgenic lines exhibited altered root architecture involving an enlarged stele and aerenchyma (Redillas et al. 2012). The second category of genes participates in the absorption and utilization of mineral elements, such as *OsPTF1*. *OsPTF1* encodes a novel transcription factor with helix-loop-helix domain. Overexpression of *OsPTF1* transgenic rice plants showed significantly higher total root length and root surface area and enhanced the tolerance to Pi starvation in rice.

Little research has been done on gene clones related to rice rooting ability. Rice rooting ability means the ability of rice to generate new roots. This depends on the number of root primordia on the stem nodes of the seedling and the nutrients provided by the plant (Dai et al. 2008). Rice rooting ability differs among rice varieties and is influenced by heterosis (Hu et al. 2004; Zheng et al. 1996). New root occurrence rating is a key factor in rice tillering (Dai et al. 2008). Plants that produce more new roots have an obvious root vigor advantage (Dai et al. 2001; Ren et al. 2007). Root growth ability and root number were confirmed to significant positively correlate with seed setting rate and 2,3,5-triphenyltetrazolium chloride (TTC) reduction ability of rice at booting stage (He et al. 2006).

Genome-wide association studies (GWAS) have been used successfully in rice to analyze the genetic basis of agronomic traits. On the basis of phenotype evaluation and genotyping, GWAS is an effective way to isolate the gene related to the target trait. For instance, researches using GWAS found that *OsSPL13* (Si et al. 2016) controls the grain length, *GSE5* (Duan et al. 2017) controls the grain width, the *bsr-d1* allele is associated with Digu Blast resistance (Li et al. 2017), *qPSR10* (Xiao et al. 2018) is related to cold tolerance, and *qTIPS-11* is associated with lateral root number (Wang et al. 2018). Recently, *OsSPY* (Yano et al. 2019), which is associated with semidwarfism and small panicles, was identified by GWAS with principal component analysis.

In our research, 145 accessions were used to conduct the GWAS. The goals were (1) to assess the natural variation for the traits related to rice seedling rooting ability in the diverse rice panel, (2) to identify the loci and candidate genes related to the rice rooting ability, and (3) to find varieties with superior rooting ability to be used in rice breeding.

Results

Population divergence and relative kinship analysis of the rice accessions

After SNP calling and filtering, 56456 SNPs distributed on all 12 chromosomes were used for the final genetic analysis. Only about 0.3% of inter-SNP distances were greater than 50 kb (Additional files 1: Figure S1A). Based on the nucleotide polymorphism, we first calculated the genetic component of each variety. The results showed that the value of Evanno's ΔK had the highest value at $K = 2$ (Fig. 1A). Therefore, combined with varieties' original information, we speculated that two subspecies, *indica* (Pop1) and *japonica* (Pop2), were present in our natural population (Fig. 1B). The neighbor-joining (NJ) tree showed the same results (Fig. 1C). Similarly, principle component analysis (PCA) results distinguished the accessions into two subpopulations, PC1 and PC2, which accounted for 39.39% and 5.57% of the genetic variation, respectively (Fig. 1D). Together, results of kinship relatedness of the pairwise accessions showed that a few accessions within Pop2 had relatively strong relatedness, while the kinship relatedness among the accessions was weak in the Pop1 (Fig. 1E). Similar results were observed from the NJ tree, in which the accessions in Pop2 showed stronger kinship relatedness than the accessions in Pop1. Moreover, the population differentiation statistics (F_{ST}) value between the two subpopulations was 0.71, indicating that a high level of subpopulation differentiation existed in the 145 accessions. Finally, we analyzed the linkage disequilibrium (LD) rate for three populations using the SNP data. The genome-wide LD decay rates of Pop1, Pop2, and the full population were estimated to be about 200 kb, 300 kb, and 600 kb, respectively (Fig. 1F). These results showed that the density of the SNP used in our study is sufficient for the GWAS.

Evaluation of the rooting ability of rice

In our research, four rooting-related traits were measured at the seedling stage to evaluate rice rooting ability. These four traits root growth ability (RGA), maximum root length (MRL), root length (RL), and root number (RN) were all close to normal distribution and had abundant variations (Fig. 2A, Additional files 1: Table S1). Among them, RGA had the highest coefficient of variation value (CV = 59.97%), followed by MRL, RL, and RN, and all of them had a CV value larger than 30% (Table 1). RN had the highest H' value of diversity index at 0.41 (Table 1), followed by RGA (0.39), MRL (0.38), and RL (0.37). These results indicated that our natural population had abundant phenotypic variations. Correlation analysis results revealed that RGA had a high positive correlation with the other three traits (Fig. 2B). The correlation coefficient between MRL and RL was the highest ($r = 0.95$). RN was moderately correlated with RGA ($r = 0.68$) and weakly correlated with RL and MRL. These results may indicate that RN is a relatively independent trait for evaluating rooting ability compared with the other traits in our research. The four traits' phenotypic variation explained by population structure (R_Q^2) were significant ($p < 0.01$), but all R_Q^2 values were relatively low. The RGA had the lowest R_Q^2 value at 0.08 (Table 1). In addition, the

comparison of the mean value of the four traits between the two subgroups (Pop1 and Pop2) by one-way analysis of variance (ANOVA) revealed obvious differences in MRL ($p = 0.0436$), RL ($p = 0.0118$), and RN ($p = 0.0133$), with the exception of RGA ($p = 0.9266$) (Additional files 1: Figure S1B). These results suggest that the effect of population structure should be considered in the next GWAS analysis.

Table 1
phenotypic variation for the root growth ability and related traits in 145 rice accessions

trait	mean \pm SD	range	CV	diversity of H' value	R_Q^2
MRL	5.59 \pm 2.63	1.50 ~ 17.77	47.05%	0.38	3.73%
RL	4.17 \pm 1.89	1.12 ~ 10.36	45.32%	0.37	5.76%
RN	4.85 \pm 1.70	1.56 ~ 12.22	35.05%	0.41	4.48%
RGA	20.76 \pm 12.45	2.34 ~ 60.47	59.97%	0.39	0.08%

GWAS analysis results for rooting ability traits in rice

Association mapping was conducted in three association panels for the full, Pop1, and Pop2 to minimize the impact of the population structure on the power of GWAS. In total, 88 ($p < 1.8 \times 10^{-5}$), 23 ($p < 2.1 \times 10^{-5}$), and 43 ($p < 3.2 \times 10^{-5}$) suggestive SNPs were detected in the full, Pop1 (Additional files 1: Figure S2), and Pop2 (Additional files 1: Figure S3) association panels for the four traits, respectively. These SNPs were distributed on all chromosomes with the exception of chromosome 10. Figure S4 clearly displayed SNP hotspots (Additional files 1: Figure S4).

Under a general linear model (GLM), the full population had most significant SNPs detected among the three panels. RN-related SNPs were detected most commonly (Fig. 3) (Additional files 2: Table S2), followed by RGA, MRL, and RL. The SNP seq4_15403602 had the lowest p -value and the highest explanation rates at 33.85%, 32.61%, and 28.92% for the RL, MRL and RGA, respectively. Additionally, there were three regions contained most of the cluster SNPs which were correlated with RN. And the lead SNPs were seq5_6945054, seq1_2164532, and seq3_35311659, respectively. Among them, the SNP seq1_2164532 had the lowest p -value and the highest explanation rate of RN variation as 17.59%.

A total of 23 significant SNPs were detected in the Pop1 among the three GWAS panels, and most of them were related to MRL, followed by RL and RGA. No significant SNP was detected for RN (Additional files 2: Table S3). The important SNP seq9_11941704, also detected in the full panel, had the lowest p -value for RL, MRL, and RGA and explained 39.33%, 47.56%, and 37.50% of phenotypic variation, respectively. Only one region containing clustering SNPs was detected for MRL, and the leading SNP seq8_18343904 could explain 43.66% of MRL variation.

In the Pop2 panel, the number of SNPs detected for RN was the highest, followed by RGA, RL, and MRL (Additional files 2: Table S4). The SNP seq4_15403602, which had been also detected in the full panel with the lowest p -value, had the highest phenotypic explanation rate for RL, MRL, and RGA in this panel. The lowest p -value SNP seq8_18343904 in this panel explained 30.20% of RN variation, and was also identified in Pop1 for MRL. In addition, there were another three regions containing the clustering SNPs with the lead SNPs seq6_1153742, seq8_15949787, and seq5_6932106, where seq6_1153742 and seq8_15949787 explained 28.15% and 32.07% of the RN variation rate, respectively, and seq5_6932106 explained 24.55% of RGA variation, which was also detected in the full panel.

Identifying the candidate genes associated with the rooting ability of rice

To obtain candidate regions from these significant SNPs, we combined the suggestively significant SNPs with quantile-quantile (Q-Q) plots of the GWAS results. We then focused on the SNPs distributed in clusters and detected by more than one panel to avoid false positives if the Q-Q plot was not well-fitted. Based on this, a total of 32 SNPs (Table 2) were obtained

Table 2
The 29 significant SNPs and their rice genomic information

Trait	marker	population-identified	allele	Chr	Pos	candidate genes
RN	seq1_2164532	full	C/T	1	2164532	<i>OsHsp17.0</i> (Ham et al, 2013)
RN	seq2_20578075	full,Pop2	T/C	2	20578075	
RN	seq3_1727891	full,Pop2	C/T	3	1727891	<i>CDPK13</i> (Ho et al, 2013)
RN	seq3_35381634	full	T/C	3	35381634	
MRL,RGA,RL	seq4_5315034	Pop2	A/G	4	5315034	<i>OsCyc1</i> (Otomo et al, 2004) <i>OsDTS2</i> (Wilderman et al, 2004)
MRL,RGA,RL	seq4_15403602	full,Pop2	T/C	4	15403602	
MRL	seq4_18740406	full,Pop1	C/G	4	18740406	<i>OsSAD5/SLL1</i> (Shelly et al, 2013)
RN,RGA	seq5_6758272	full	T/C	5	6758272	<i>OsPYL</i> (Kim et al, 2014);
RN,RGA	seq5_6784506	full	A/G	5	6784506	<i>OsGA2ox10</i> (Fang et al, 2008)
RN,RGA	seq5_6784672	full	A/C	5	6784672	
RGA	seq5_6882162	full,Pop2	T/C	5	6882162	
RGA	seq5_6884530	full,Pop2	C/A	5	6884530	
RGA	seq5_6890004	full,Pop2	G/A	5	6890004	
RGA	seq5_6908893	full,Pop2	T/C	5	6908893	
RGA	seq5_6935074	full,Pop2	T/G	5	6935074	
RN,RGA	seq5_6945054	full,Pop2	T/C	5	6945054	
RGA	seq5_6959356	full,Pop2	G/A	5	6959356	
RGA	seq5_6981293	full,Pop2	T/C	5	6981293	
RN	seq6_11153742	full,Pop2	C/T	6	11153742	
MRL,RL	seq6_12537542	full,Pop2	T/C	6	12537542	<i>OsPT9</i> (Wang et al, 2014)

Trait	marker	population-identified	allele	Chr	Pos	candidate genes
MRL,RL	seq6_29405495	full,Pop1	C/T	6	29405495	<i>OsMPK4</i> (Agrawal et al, 2003)
MRL,RL,RGA	seq7_5182658	full,Pop2	A/G	7	5182658	<i>SQS</i> (Manavalan et al, 2012)
MRL,RL,RGA	seq8_433198	full,Pop1	G/T	8	433198	
RN	seq8_15949787	Pop2	G/C	8	15949787	<i>qtl</i> (Lilley et al, 1996)
MRL,RN	seq8_18343904	Pop1,Pop2	T/G	8	18343904	
MRL,RN	seq8_18364190	full,Pop1,Pop2	C/T	8	18364190	
MRL	seq8_18365309	full,Pop1	C/T	8	18365309	
MRL,RL,RGA	seq9_11941704	full,Pop1	T/C	9	11941704	
MRL,RL	seq9_15520580	Pop1	A/C	9	15520580	<i>OsGL1-1</i> (Islam et al, 2009)

Traditionally speaking, the gene nearest to SNP is not always the causal gene. Therefore, we screened the candidate genes from the LD blocks mentioned according to their annotation information from the China Rice Data Center (<http://www.ricedata.cn/gene/>) and the Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/index.shtml>). We mainly selected genes with GO information related to metabolic processes, catabolic processes, or stress responses or genes that have a high expression level at seedling stages. As a result, some known genes and QTLs reported previously were obtained (Table 2). By summarizing the given annotation information, these genes and QTLs could be classified into four categories. The first category included six genes related to endogenous hormone metabolism. *Asr1*, located at about 310 kb from the SNP seq11_3585036, encodes an abscisic acid-stress-ripening-inducible 5 protein, and is regulated by both abscisic acid and gibberellin (Takasaki et al. 2008), which is also involved in drought tolerance (Philippe et al. 2010). *OsCDPK13* was located at about 99 kb upstream of the SNP seq3_1727891. It has been found to negatively regulate the expression level of the enzymes essential for gibberellin biosynthesis and prevent drought stress injuries in the seedling development stage (Ho et al. 2013). *OsMPK4* was mapped at about 4 kb upstream of the SNP seq6_29405495. Its transcript's level is up-regulated upon wounding (by cut), hormones, and heavy metals, et al (Agrawal et al. 2003). In the second category, two genes are related to phosphate uptake. *OsMYB2P-1* was identified at about 106 kb upstream of the SNP seq5_2421894. Longer primary roots and adventitious roots were produced in the *OsMYB2P-1*-overexpression plants than in wide-type plants under Pi-deficient conditions (Dai et al. 2012). *OsPT9* was identified at about 142 kb downstream of the SNP seq6_12537542. It encodes a phosphorus transporter and its expression is specifically induced by Pi starvation (Wang et al. 2014). In the third category, two genes were responsible for root drought tolerance. *OsHSP17.0* was found at about 3 kb from the nearest SNP, seq1_1954975. Overexpression *OsHSP17.0* transgenic lines plants

displayed a higher tolerance to drought and salt stress compared to wild-type plants (Ham et al. 2013). *OsGL1-1* was located at 24 kb upstream of the nearest SNP seq9_15520580. *OsGL1-1* is a Glossy1-homologous gene and *osgl1-1* mutants show increased sensitivity to drought (Islam et al. 2009). In the fourth category, two genes were correlated with morphogenesis of roots. *SQS* and *SLL1* directly influence root architecture and were found at 257 kb downstream of the SNP seq7_5182658 and 348 kb upstream of the SNP seq4_18740406, respectively. RNAi of the *SQS* (farnesyltransferase/squalene synthase) plants grown in plates with Yoshida's nutrient solution with 1.2% agar show increased root length and an enhanced number of lateral roots (Manavalan et al. 2012). Over-expression *SLL1* plants produced significantly longer lateral roots compared to wild-type plants (Shelley et al. 2013). Meanwhile, one region containing the lead SNP seq8_15949787 belonged to a previously reported QTL (Lilley et al. 1996) of root dehydration tolerance.

In total, 26 genes were used to conduct the quantitative real-time PCR (qRT-PCR). The primers used for the qRT-PCR are listed in the supplementary materials (Additional files 2: Table S5). Considering these loci responsible for different traits and the correlation among them, we mainly chose varieties with contrasting RN or RL. Taking into consideration that these two traits exhibit differences between the *indica* and *japonica* varieties, we mainly choose the varieties within the same subpopulation. More importantly, we also sequenced possible genes in varieties with contrasting phenotypes to find possible excellent haplotypes, taking into consideration the fact that few genes had low expression levels in roots and proved to be outstanding candidate genes for improved abiotic stress resistance. Interestingly, we not only found two genes that differ in relative expression level between differing RLs (Additional files 2: Table S6), but we also found that one gene's sequence variation is associated with RN. The results are as follows.

One candidate region *qRL-4* of about 480 kb for RL, MRL, and RGA including the significant SNP is located at about 5.3 Mb on chromosome 4 (Fig. 4A). In *qRL-4*, we determined that *LOC_Os04g09900* (*OsCyc1*) encodes syn-copalyl diphosphate (syn-CDP) synthase, which is responsible for phytoalexin biosynthesis (Otomo et al. 2004). A comparison of expression levels of this gene among the 10 accessions with extreme RL differences ($p < 0.01$) was conducted by qRT-PCR. *LOC_Os04g09900* had a higher expression level in the group with longer root length (LRL group) than the group containing varieties with shorter root length (SRL group) ($p = 0.0330$) (Fig. 5). Another gene, *LOC_Os04g10060* (*OsDTS2*), encodes 9 β -pimar-7,15-diene synthase. Previous qRT-PCR results (Wilderman et al. 2004) showed that varieties whose root length was longer had higher expression level. Another region, *qRN-5*, for RN and RGA is about 300 kb. It contains a cluster of significant SNPs and the lead SNP is seq5_6945054 (Fig. 4B). Two known genes, *LOC_Os05g11810* (*OsGA2ox10*) and *LOC_Os05g12260* (*OsPYL*), were identified in *qRN-5*. *LOC_Os05g11810* encodes the gibberellin 2-oxidases (GA2oxs), which can regulate plant growth by inactivating endogenous bioactive gibberellin (GA) (Lo et al. 2008), and *LOC_Os05g12260* encodes a rice orthologue of the ABA receptor (Kim et al. 2014). From the sequencing results, we only found sequence variations of *LOC_Os05g11810* among the varieties with root number differences (Fig. 6). Based on these, we divided varieties into two haplotypes (hap1 and hap2). Hap2, sharing the same haplotype with nipponbare rice, had fewer roots compared with hap1 ($p =$

0.0005). Additionally, both of the two groups had low transcripts which may cause that although hap2 group had a higher mean value of relative expression level than hap1, it was not statistically significant ($p = 0.3727$).

Discussion

Characterization of the rooting ability in rice seedlings

A vigorous root system is the basis of vigorous growth in rice at early stage and grain filling at later stage. Furthermore, rooting ability was confirmed to be inherited and showed significant heterosis (Zheng et al. 1996). Rice rooting ability is determined by a number of parameters including seedling leaf age and seedling quality. Based on previous research, a nutrient solution was used for cultivating root-cut seedlings to provide a consistent nutritional environment to minimize the impact of the upper part on root systems. Simultaneously, we evaluated the rooting ability of rice at the seedling stage by measuring the morphological indicators of root systems such as root length and root number. The four evaluated traits showed abundant variation and continuous distribution, suggesting that these traits related to rooting ability are quantitative traits and suitable for GWAS.

From the comparison of phenotypic traits in different populations, there were certain phenotypic differences between *indica* and *japonica* varieties. RN, RL, and MRL showed few differences at the significant level of $p < 0.05$ in the two subpopulations, while the difference of RGA between the two subpopulations was not significant. Similar results were also obtained from the analysis of the phenotypic variation explained by population structure, in which RGA have the lowest R_Q^2 value. This indicated that *japonica* tends to possess longer root length and *indica* tend to possess larger number of roots when their roots were cut at the seedling stage. This may be due to the different ecological environmental conditions of subpopulations. *Japonica* is generally distributed in temperate regions, high latitude regions, tropical and subtropical mountains, and arid regions. Rice plants in these regions require longer root length to absorb water. *Indica* is usually distributed in low latitudes and tropical and subtropical lowlands of China and Southeast Asia. The abundance of water in these regions may cause these varieties to have a higher number of relatively shallow roots and stronger tillering (Xu et al. 2012). This is consistent with the result of a previous study on rice roots (Zhao et al. 2019). There was no significant difference in the final RGA between the two subpopulations. It suggests that both two subpopulations possess considerable ability for rooting prior to root damage despite their differing root morphology.

Correlation analysis showed that RGA positively related with RL, MRL, and RN. By contrast, the correlation between RL and RN is weak. This may indicate that the genetic basis for RN is relatively independent from RL. These results corresponded to the association results, in which only one significant SNP could be detected for multiple traits.

GWAS results analysis among different association panels

GWAS is an efficient way to analyze genetic variation for multiple traits in rice (Lu et al. 2016; Zhang et al. 2018). In GWAS, accuracy and precision are influenced by population structure, kinship, and LD decay rate.

In the present study, LD decay distance results showed that the full population had the highest LD decay distance. Similar results were reported in a previous study, suggesting that population mixing could introduce chromosomes from different ancestral sources and allele frequencies to affect LD level (Wang et al. 2007). Population structure analysis divided the full population into two distinct subpopulations. This result was supported by the results of PCA and the NJ tree. In addition, the distance matrix and NJ tree showed that only a few varieties in Pop2 have close kinship while the majority Pop1 varieties showed evidence of weak kinship relatedness. Thus, to avoid overcorrection, we only used the Q matrix as the covariate for GWAS in three panels. Furthermore, the p -value in the GLM model was close to the expected p -value, which indicated that the GLM model is suitable.

In our GWAS results, there are differences in the suggestive loci for different traits and different populations. There were some SNPs detected for more than one trait, which could be explained by pleiotropic genes. Many significant SNPs related to RGA could also be detected in GWAS for RN or RL, and similarly, MRL and RL also shared the same SNPs discovered by GWAS. In contrast, there were SNPs identified in more than one GWAS panel. In most cases, the SNPs discovered in the full population were also could be detected by the Pop1 or Pop2 panel. However, some SNPs could only be detected in the Pop1 or Pop2 association panel, which may be due to the low frequency of rare SNPs. As for some significant SNPs only detected in full association panel, which may be due to false positive caused by population structure. In addition, most loci related to RN were detected in *japonica*, while most loci related to MRL were detected in *indica*. This may be because *indica* and *japonica* have different domestication processes, leading to different phenotypic variations and different genetic backgrounds in the two subpopulations. There was only one candidate region including two SNPs detected simultaneously in the two subpopulations. This may reflect the considerable differences in the genetic background of the *indica* and *japonica* for rooting ability. The strong heterosis of rice rooting ability may be elucidated by the recombination of the positive loci in different subpopulations.

Analysis of the regulatory mechanism of participating in rooting ability

According to the GWAS results, the association study was efficient in determining the number of loci controlling rice rooting ability, enabling us to identify a series of cloned genes from the candidate regions. Through summarizing the functions of these known genes, our data suggested that hormone signaling pathways (*Asr1*, *CDPK13*, *OsARF4* and *OsMPK4*), phosphorus absorption pathways (*OsMYB2P-1* and *OsPT9*), and the mechanism of both root morphology (*SQS* and *SLL1*) and drought-tolerance (*OsHSP17.0*

and *OsGL1-1*) may regulate rice rooting ability. These results may be illustrated by the fact that cutting roots causes nutrient and water deficiency and new roots are needed to absorb both nutrients and water. The results could also suggest that endogenous hormone metabolism could regulate the growth of new roots, which is consistent with previous research that reported that the content of endogenous hormones in seedlings can affect the RGA of seedling rice. Specifically, endogenous hormone abscisic acid in roots can inhibit RGA, while endogenous hormone gibberellin can promote RGA (Ren et al. 2009). These results may provide us a perspective on rooting vigor breeding by aggregating the genes from these four pathways. However, further research will be necessary to confirm this.

Identification of candidate genes controlling the root growth ability of rice

Here, we identified three candidate genes that may be responsible for rice rooting. For the candidate genes identified by qRT-PCR analysis, both *OsCyc1* and *OsDTS2* had expression level differences for contrasting phenotypes. *OsCyc1* is responsible for phytoalexin biosynthesis, and its transcript level increased after infection by rice blast (Otomo et al. 2004). *OsDTS2* (Wilderman et al. 2004) encodes a syn-copalyl diphosphate specific 9 β -pimara-7,15-diene synthase. Its mRNA level in leaves is up-regulated by stimulating phytoalexin biosynthesis but is constitutively expressed in roots. Both of them had higher expression levels in groups with longer root length than in groups with shorter root length; thus, we inferred that cutting roots caused abnormal growth and that varieties with more transcripts could produce longer roots for absorbing water and nutrients. Furthermore, both genes are involved in allelopathy which were benefit for rice plants in suppressing growth of widespread rice paddy weeds. This is consistent with previous research that vigorous root systems in rice enhance competitiveness with weeds.

However, DNA sequence analysis showed that one gene's sequence variations were closely associated with phenotype. We concluded that *OsGA2ox10* may be responsible for root number variation and the hap2 of the *OsGA2ox10* is the positive haplotype. In Lo's research (Lo et al. 2008), *OsGA2ox10* was not detected the mRNA level, while the expression of the *OsGA2ox10* in our research was detected in roots, though the expression level was relatively low. Despite the relative expression and haplotype analysis verification, these three genes need to be verified using further genetic complementation experiments.

Conclusion

In our study, we dissected the genetic basis of the rooting ability of rice at the seedling stage in *indica* and *japonica* rice subgroups. This provides valuable information for future study on the genetic basis of rice rooting ability. We also identified some candidate regions and genes that are related to rooting ability. Moreover, the SNPs found in these regions and genes could be used for future gene validation and marker-assisted selection. Our results may provide useful information for rice root breeding.

Materials And Methods

Plant materials

A panel consisting of 145 accessions was used for GWAS. These 145 rice varieties included both *indica* and *japonica* varieties, and most of them were collected from the south of China. To ensure homogeneity, they were grown at the experimental farm of the China National Rice Research Institute in 2016 and 2017. Plant density was four lines per plot with six individuals per line. At harvest, the seeds were collected from the middle plants in each line.

Phenotypic evaluation at the seedling stage

After being fully air-dried, the seeds were soaked for two days in a germination-accelerating solution for 12 h at 30 °C. They were then sown in the soil at the green house. Three weeks later, the crown roots of each accession were cut off. The plants were then transferred into glass tubes filled with Yoshida nutrient solutions (Yoshida et al. 1971). Three single plants of each accession were fixed by black sponge in one glass tube for three replications in total. The glass tubes were surrounded by the black cloth to block direct light. Nutrient solutions were changed every three days. One week after root cutting, the number and length of new roots were counted and measured by ruler for three single plants per replication. Finally, four rooting-related traits were evaluated: RN, RL, MRL, and RGA. Both the RN and MRL were calculated by averaging the values for all plants in three replications. To find the RL, we first calculated the average value of the RL for a single plant. We then computed the mean RL of the plants belonging to one accession. To find the RGA, the RGA of a single plant was calculated using the formula: $RGA = RN \times RL$ (mean of the root length per plant). The average of the total single plant's RGA for one accession was used as the final RGA for the GWAS analysis.

Phenotypic data analysis

Mean, standard deviation, and diversity index of the H' value were calculated in EXCEL 2010. The mean and standard deviation were calculated using the AVERAGE () and STDEVP (). Based on the mean value and standard deviation of each trait, we divided 145 accessions into 10 levels, from the first level ($X_i < X - 2\sigma$) to the tenth level ($X_i > X + 2\sigma$). Every 0.5σ was a level. According to these grading results, the modified Shannon-Wiener diversity index was used to calculate the diversity index of different traits. The calculation formula is: $H' = (-\sum P_i \ln P_i) / \ln N$, where H' represents the diversity index, P_i is the percentage of varieties in the i_{th} grade of one trait in the total number of varieties in one period, and N is the total number of varieties in the period. The percentage of phenotypic variation explained by the population structure (R_Q^2) was analyzed using a one-way analysis of variance (ANOVA) in the SAS 9.4 system. Correlation analysis of traits was conducted by the package "corrplot" in R (3.6.0). ANOVA conducted in the EXCEL 2010 was separately used to test significant phenotypic differences between the two subpopulations. Additionally, "two sample t -test for means" in the SAS system was used to analyze the

phenotypic or relative expression level differences between the varieties with a contrasting phenotype or haplotype.

GWAS analysis

About 65 K SNP genotype data of the 145 accessions were obtained from a 90 K high-density SNP array. A total of 56456 SNPs with minor allele frequency less than 0.05 and a minimum count less than 75% of total individuals filtered by TASSEL 5.2.51 (Bradbury et al. 2007) were used for association analysis of the full panel. Population structure analysis was conducted using the STRUCTURE software (2.3.4), and the Nei's genetic distance's calculation and neighbor-joining (NJ) tree construction were both conducted by PowerMarker version 3.25. Both the principle component analysis (PCA) and genome linkage disequilibrium (LD) analysis were conducted by the Tassel (5.2.51). The LD decay rate was measured as the physical distance between SNPs at which the average pairwise correlation coefficient (r^2) went down to the half of the maximum (Huang et al. 2010). The distance matrix calculated by Tassel (5.2.51) was used to evaluate the kinship relatedness of the pairwise accessions. A total of 56456 SNPs (full), 47984 SNPs (Pop1), and 31646 SNPs (Pop2) after filtering were used for association mapping according to a general linear model (GLM) using the Q matrix as the covariate. Additionally, based on the Bonferroni-corrected threshold setting the effective number of independent makers at a significant level of 1, we set the p threshold qualification as $1/n$ (n = the number of SNP). For clustered significant SNPs within the LD decay distance of the whole genome, we chose the SNP with lowest p value as the lead SNP. LD block was calculated using the Haploview 4.2 to obtain the candidate regions.

Quantitative real-time PCR and sequence analysis

Total RNA extraction from the plant's seedling roots used the Total RNA extraction kit. First-strand cDNA was synthesized using PrimerScript RT Master Mix. Then, quantitative real-time PCR (qRT-PCR) was performed in a two-step reaction using PowerUp SYBRGreen Master Mix on an Applied Biosystems 7500 Real-Time PCR system. The rice ubiquitin gene was used as the internal control. Each measurement was performed on three replicates of each of three biological samples. The sequences of the candidate genes were downloaded from the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/analyses_search_blast.shtml). The 50 μ l total PCR reaction volume contained 25 μ l KOD FX PCR buffer, 10 μ l dNTPs, 1.5 μ l each of 10 pmol/ μ l primer, 5 μ l template DNA, 6 μ l PCR grade water, and 1 μ l KOD FX. The PCR cycle conditions were as follows: initial incubation at 94 °C for 2 min, 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 68 °C at 1 kb/1 min, followed by a final extension at 68 °C for 8 min.

Abbreviations

GWAS: genome-wide analysis study

LD: Linkage disequilibrium

MRL: maximum root length

PCA: Principle component analysis

qRT-PCR: quantitative real-time Polymerase Chain Reaction

RGA: root growth ability

RN: root number

RL: root length

SNP: Single nucleotide polymorphism

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for Publication

Not applicable.

Availability of Data and Materials

All data supporting the conclusions of this article are provided within the article (and its Additional files).

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

XX and XH designed and supervised the researches. JH, YY and MC performed the experiments. QX, YF, YP, XP and HY analyzed the data. XX, YY and XH wrote the manuscript. All authors read and approved the final manuscript.

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Figures

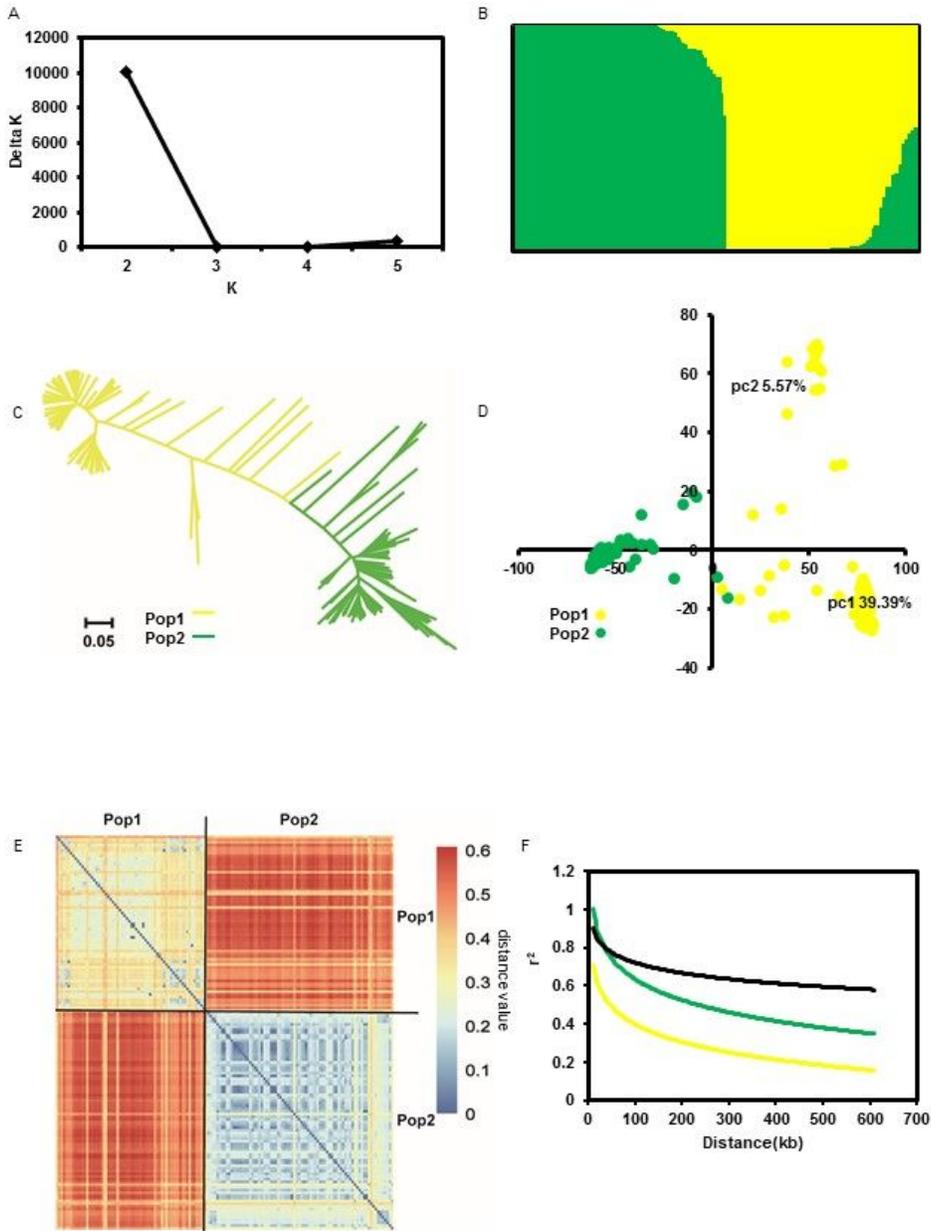


Fig.1

Figure 1

(a) Delta K values plotted as the number of subpopulations. (b) Subpopulations (K = 2) inferred using STRUCTURE. (c) NJ tree based on Nei's genetic distances. Black, yellow, green represent full, Pop1, and Pop2, respectively. (d) Principal component analysis of rice panel. Values in parentheses indicate the percentage of variance in the data explained by each principal component. (e) Pairwise relative kinship

analysis by distance matrix of rice panel. (f) Genome-wide average LD decay estimated from 145 full (black), 56 Pop1/indica (yellow), 89 Pop2 / japonica (green) landraces.

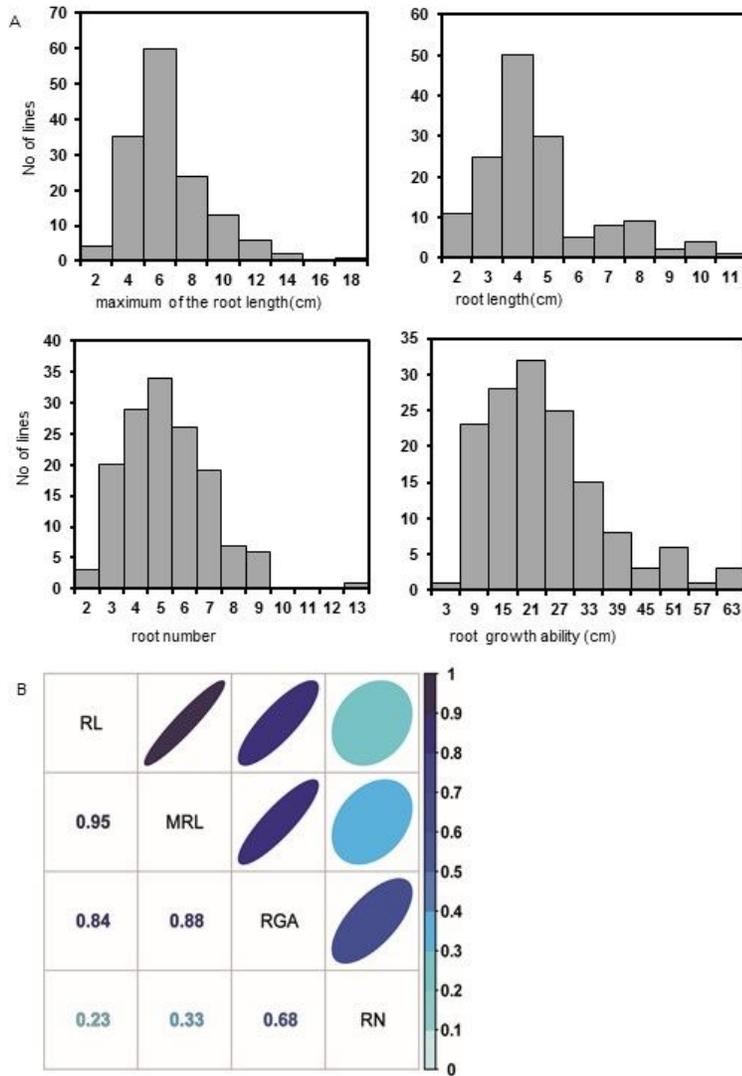


Fig.2

Figure 2

(a) Phenotypic distribution of root-related traits in the natural population. (b) Correlation analysis of root-related traits. The color and area of the circle represent the value of the correlation coefficient. The number in the middle of the cell is the correlation coefficient.

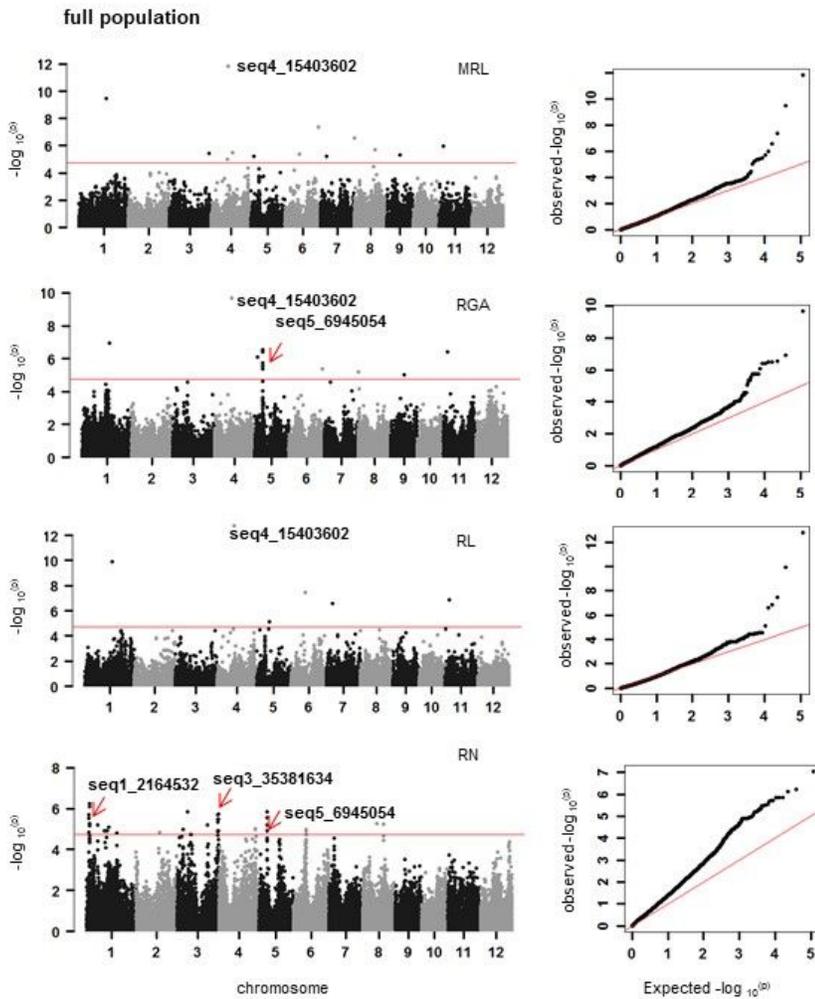


Fig.3

Figure 3

Manhattan plots and quantile-quantile (Q-Q) plots of GWAS for root growth ability, maximum root length, root length, and root number of the full association panel. The red line in the Manhattan plot represents the significance threshold. The red straight line in the quantile-quantile (Q-Q) plot represents expected null distribution of p-value and black dots represent observed distribution of p-values.

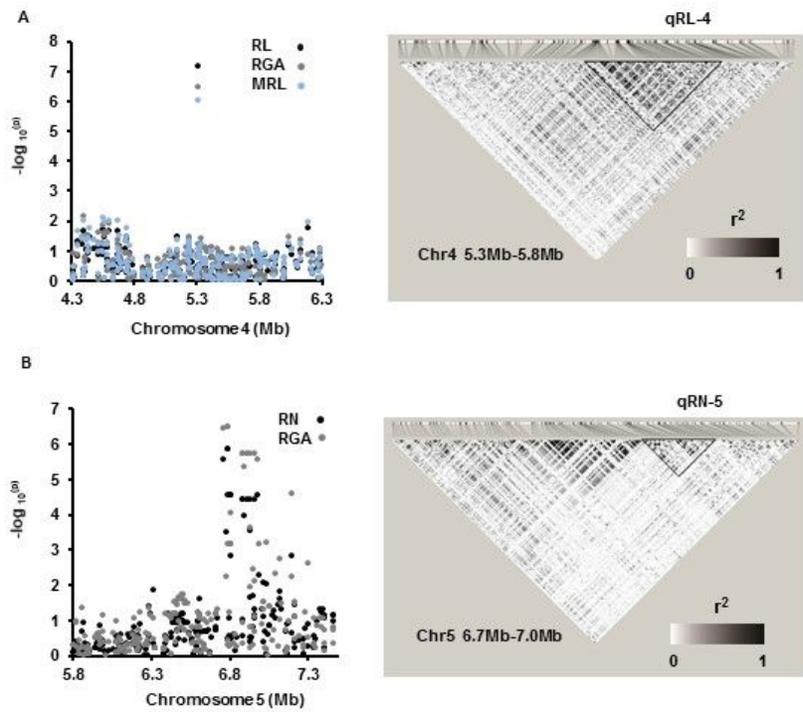


Fig.4

Figure 4

(a) Local Manhattan plots (left) of GWAS for maximum root length, root length, and root growth ability and the Pop2 association panel and linkage disequilibrium heatmap (right) surrounding the peak on Chromosome 4. (b) Local Manhattan plots (left) of GWAS for root growth ability and root number for the full association panel and linkage disequilibrium heatmap (right) surrounding the peak on Chromosome 5.

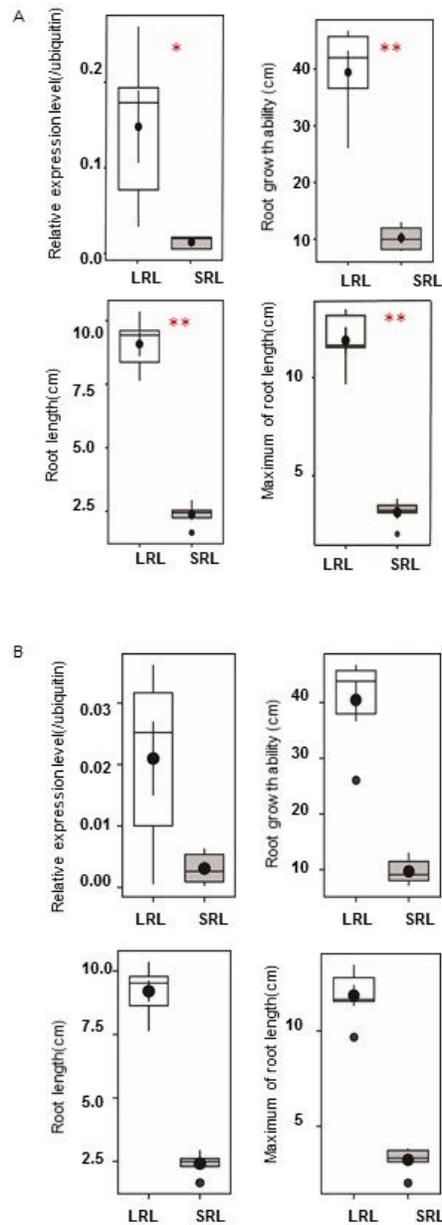


Fig.5

Figure 5

(a) Boxplots of relative expression level, root number, maximum root length, and root growth ability in different groups with contrasting phenotypes of LOC_Os04g09900 (OsCyc1). LRL means varieties in the group with longer root length and SRL means the varieties in the group with shorter root length. Box edges represent the 0.25 and 0.75 quantiles with the median values shown by bold lines. Whiskers extend to data no more than 1.5 times the interquartile range and the remaining data are indicated by asterisks.

“*” on the top of the boxplots represents the significance at 0.05 probability levels and “**” represents the significance at 0.01 probability levels. (b) Boxplots of relative expression level, root number, maximum root length, and root growth ability in different groups with contrasting phenotypes of LOC_Os04g10060 (OsDTS2).

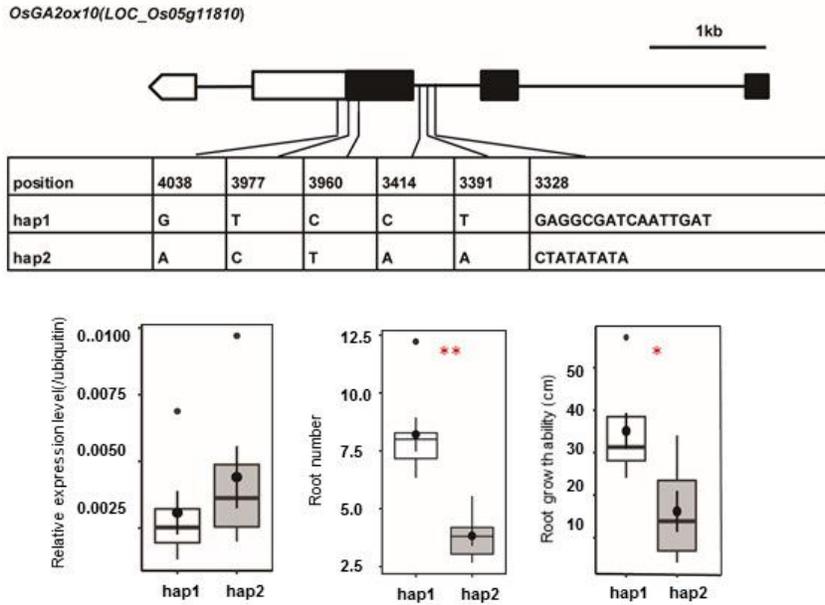


Fig.6

Figure 6

Gene structure of the candidate gene LOC_Os05g11810(OsGA2ox10); the solid box and empty box indicate the exon and untranslated region, respectively. Boxplots of relative expression level, root number and root growth ability in different haplotypes of LOC_Os05g11810(OsGA2ox10).

Supplementary Files

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