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# Linkage Mapping and Discovery of Candidate Genes for Drought Tolerance in Rice During the Vegetative Growth Period

Aixia Jiao<sup>1,2†</sup>, Li Chen<sup>4†</sup>, Xiaoding Ma<sup>2,3</sup>, Jing Ma<sup>4</sup>, Di Cui<sup>2,3</sup>, Bing Han<sup>2,3</sup>, Jianchang Sun<sup>4\*</sup> and Longzhi Han<sup>2,3\*</sup>

## Abstract

Drought is a major abiotic stress affecting crop yields. Mapping quantitative trait loci (QTLs) and mining genes for drought tolerance in rice are important for identifying gene functions and targets for molecular breeding. Here, we performed linkage analysis of drought tolerance using a recombinant inbred line population derived from Jileng 1 (drought sensitive) and Milyang 23 (drought tolerant). An ultra-high-density genetic map, previously constructed by our research team using genotype data from whole-genome sequencing, was used in combination with phenotypic data for rice grown under drought stress conditions in the field in 2017–2019. Thirty-nine QTLs related to leaf rolling index and leaf withering degree were identified, and QTLs were found on all chromosomes except chromosomes 6, 10, and 11. *qLWD4-1* was detected after 32 days and 46 days of drought stress in 2017 and explained 7.07–8.19% of the phenotypic variation. Two loci, *qLRI2-2* and *qLWD4-2*, were identified after 29, 42, and 57 days of drought stress in 2018. These loci explained 10.59–17.04% and 5.14–5.71% of the phenotypic variation, respectively. There were 281 genes within the QTL interval. Through gene functional annotation and expression analysis, two candidate genes, *Os04g0574600* and *OsCHR731*, were found. Quantitative reverse transcription PCR analysis showed that the expression levels of these genes were significantly higher under drought stress than under normal conditions, indicating positive regulation. Notably, *Os04g0574600* was a newly discovered drought tolerance gene. Haplotype analysis showed that the RIL population carried two haplotypes (Hap1 and Hap2) of both genes. Lines carrying Hap2 exhibited significantly or extremely stronger drought tolerance than those carrying Hap1, indicating that Hap2 is an excellent haplotype. Among rice germplasm resources, there were two and three haplotypes of *Os04g0574600* and *OsCHR731*, respectively. A high proportion of local rice resources in Sichuan, Yunnan, Anhui, Guangdong and Fujian provinces had Hap of both genes. In wild rice, 50% of accessions contained Hap1 of *Os04g0574600* and 50% carried Hap4; 13.51%, 59.46% and 27.03% of wild rice accessions contained Hap1, Hap2, and Hap3, respectively. Hap2 of *Os04g0574600* was found in more *indica* rice resources than in *japonica* rice. Therefore, Hap2 has more potential for utilization in future drought tolerance breeding of *japonica* rice.

<sup>†</sup>Aixia Jiao and Li Chen contributed equally to this work.

\*Correspondence:  
Jianchang Sun  
nxsjch@163.com  
Longzhi Han  
hanlongzhi@caas.cn

Full list of author information is available at the end of the article

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## Background

Drought tolerance is a complex plant abiotic stress response trait. Researchers worldwide have conducted extensive research on rice drought tolerance in terms of morphology, physiology, biochemistry, and molecular biology, with significant results. These studies have been instrumental in the development of strategies for breeding drought tolerance in rice. However, there remains a sizable disparity between current achievements and desired goals. Rapid advances in modern biotechnology have facilitated the application of tools, such as linkage and association analyses based on genomics data, in studies of fundamental trait genes. These studies have revealed the molecular mechanisms underlying plant responses to adverse abiotic stresses and opened new paths for enhancing crop tolerance to these stresses.

Over the past 30 years, researchers have used tools such as linkage analysis combined with molecular marker analysis and genome sequencing to systematically study drought tolerance and related traits of rice at different growth stages and in various populations. Vikram et al. (2011) and Ghimire et al. (2012) used linkage analysis to identify a key drought-tolerance QTL, *qDTY<sub>1.1</sub>*, that affects yield. This QTL was mapped to the same chromosome location in diverse study populations. Yadav et al. (2019) used high-density linkage maps for two BC<sub>1</sub>F<sub>3</sub> genetic populations, Swarna\*2/Dular and IR11N121\*2/Aus 196, which they constructed utilizing genotyping-by-sequencing data, to identify six and eight QTLs, respectively. Notably, two of these QTLs (*qDTY<sub>2.4</sub>* and *qDTY<sub>3.3</sub>*) were mapped to the same chromosomal region as the known drought tolerance QTL *qDTY<sub>1.1</sub>*. Pan et al. (2021) identified five QTLs related to leaf water potential at the tiller stage, each located on a different chromosome, using a recombinant inbred line (RIL) population (Nekken2/HZ). Chen et al. (2023) mapped 28 QTLs related to leaf rolling index (LRI), leaf withering degree (LWD), and leaf chlorophyll content (LCC) utilizing a RIL population and simple sequence repeat (SSR) markers. The QTLs identified from these studies have been crucial for understanding the hereditary foundation of drought tolerance in rice. These studies have also provided essential genetic information for the design of molecular markers for marker-assisted selection (MAS) breeding, transgenic analysis, and gene editing. For example, researchers have used QTLs related to traits such as root system architecture and yield for MAS breeding of rice varieties with superior yield and drought tolerance (Mishra et al. 2013; Catolos et al. 2017; Yadav et al. 2019). Furthermore, researchers have made considerable progress in the map-based cloning and functional studies of drought-tolerance

genes (Zhu and Xiong 2013; Chen et al. 2014; Geng et al. 2023), including *DRO1*, *OsZIP46*, *DROT1*, and *SANCI* (Uga et al. 2013; Tang et al. 2016; Sun et al. 2022; Gao et al. 2023). Using the fun Rice Genes database (<https://fun-ricegenes.github.io>), researchers have successfully cloned 378 genes related to rice drought tolerance. However, the number of cloned genes is relatively small compared with the number of QTLs that have been mapped. This could be attributed to the low logarithm of the odds (LOD) scores and phenotypic contribution rates of several QTLs, or the variability in performance across diverse environments and the wide confidence intervals (Geng et al. 2023). Therefore, it is critical to identify reliable and dominant QTLs using high-density genetic maps and accurate phenotypic data.

In this study, we used an RIL population that was established by crossing the drought-sensitive cultivar Jileng 1 (JL1) and the drought-tolerant cultivar Milyang 23 (MY23). QTLs were mapped using an ultra-high-density genetic map and drought resistance phenotypes that were accurately measured over multiple years. The candidate genes for drought tolerance QTLs were verified by quantitative reverse transcription PCR (qRT-PCR) and haplotype analysis. Excellent haplotypes related to rice drought tolerance were then selected. This study provides valuable genetic resources for exploring the functions of rice drought tolerance-related genes and promoting the molecular breeding of rice drought tolerance.

## Materials and Methods

### Plant Materials

The research material used was a RIL population made by crossing JL1 (female parent) and MY23 (male parent). The population consisted of 253 RILs obtained using the single seed descent (SSD) method. JL1, a *japonica* rice variety, was drought-sensitive throughout the growth period, while MY23, an *indica* rice variety, exhibited strong drought tolerance throughout the growth period.

### Phenotypic Analysis of Drought Tolerance

From 2017 to 2019, tests were conducted at the Wanghong Experimental Base at the Crop Research Institute of Ningxia Academy of Agriculture and Forestry Sciences to phenotype the drought-tolerant parents and 253 RILs. The area is dry and there is little rainfall. Yinchuan receives an average annual precipitation of about 200 mm. In 2017, seedlings were sown on April 28 and transplanted in the experimental field on May 30. Irrigation was stopped on June 10, and LRI and LWD were observed on days 32 (July 11) and 46 (July 25) of the drought treatment. In 2018, the seedlings were sown

on April 25 and transplanted in the field on May 21. The water supply was cut off on June 1. LRI and LWD were measured on days 29 (June 29), 42 (July 12), and 57 (July 27) of the drought stress. In 2019, the seedlings were sown on April 23 and transplanted in the field on May 25. Irrigation was discontinued on June 5. LRI and LWD were measured on 33 (July 7), 49 (July 23), and 63 (August 6) of the drought treatment. The criteria used for scoring LRI and LWD are listed in Table 1. A smaller LRI and LWD indicates higher drought tolerance. The field experiment followed a random block design, with each material planted in two rows of 10 plants each, and a single plant was transplanted in each hole. The plant spacing was 9.9 cm, and the row spacing was 26.7 cm. Pest control and weeding were performed in accordance with local field practices.

### Linkage Analysis

We previously created an ultra-high-density genomic map for rice using whole-genome sequencing data (Geng et al. 2023). The genotypic data were combined with phenotypic data from three consecutive years of measuring drought tolerance in the field, and the Inclusive Composite Interval Mapping (ICIM) model in IciMapping v4.2 software was used to fine-map QTLs for LRI and LWD and to assess the extent to which each QTL explained the phenotypic variation. The mapping was performed with a scanning step of 1 cM and a LOD threshold of  $\geq 2.5$  for determining significance. QTLs were named following the principles proposed by McCouch et al. (1997). In cases where QTLs controlled different traits within the same interval, it was envisaged a co-localization phenomenon.

### RNA Extraction and qRT-PCR Analysis

Seven drought-tolerant lines were selected from the RIL family groups and analyzed for expression of genes related to drought tolerance. After germination, 30 seeds

per line were sown in a seedling tray (length  $\times$  width  $\times$  height = 54 cm  $\times$  28 cm  $\times$  5 cm) and placed in an incubator at 28 °C for 4 days. The seedlings were then transplanted into two plastic boxes (length  $\times$  width  $\times$  height = 60 cm  $\times$  45 cm  $\times$  20 cm) with a soil depth of 18 cm, with separate treatment and control groups. After 21 days of seedling growth, the treatment group was subjected to drought stress by stopping irrigation, while the control group was provided ample water. Fresh leaves were cut from both groups when the soil water content in the treatment group reached 14%. The leaves were quickly frozen in liquid nitrogen and kept at -80 °C. RNA was extracted using the TRIzol reagent, and 1  $\mu$ g of RNA was used for reverse transcription. qPCR was performed on the StepOne Plus real-time quantitative PCR instrument (ABI Company) with a reaction system of 20  $\mu$ L. The sequence-specific primers used are shown in Supplementary Table 1. The qPCR procedure consisted of two steps: denaturation for 30 s at 95 °C, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s. The relative gene expression was calculated using the  $2^{-\Delta\Delta CT}$  method with UBI as the reference gene (Livak et al. 2001). Three biological replicates were performed.

### Haplotype Analysis

To investigate the markers associated with QTLs and the superior haplotypes within the identified regions, the current paper utilized PLINK software for genotype data analysis. We obtained 1,860,935 high-quality SNP markers by excluding those with allele frequencies below 0.05. After filtering, we obtained a representative set of SNPs, which were used for additional haplotype analysis. The filtered SNP data were converted to Variant Call Format (VCF) utilizing PLINK software. The genotype data from the VCF file were then imported into Haploview 4.2 software for haplotype analysis. To investigate the relationship between haplotype and drought tolerance traits, analysis of variance (ANOVA) was performed using SPSS 27.0 software. The phenotypic data for each line were used along with haplotype data to identify significant haplotypes associated with superior drought tolerance traits.

## Results and Analysis

### QTL Mapping of LRI and LWD under Drought Stress

In 2017, six QTLs related to LRI were identified on chromosomes 1, 4, 5, and 7. The most significant QTL (LOD value = 20.73) detected on day 32 of drought stress was *qLR15-2* on chromosome 5 (Table 2). This QTL explained 10.34% of the phenotypic variation and had the largest effect size. Three QTLs showed positive additive effects, indicating that the beneficial alleles came from the male parent MY23. Three QTLs showed negative additive effects, indicating that the beneficial alleles originated

**Table 1** Visual evaluation criteria for LRI and LWD

Level	LRI under drought stress	Level	LWD under drought stress
1	leaves slightly	1	No symptoms; sharp leaves slightly dead
3	Leaves beginning to appear curly, folded (V shaped)	3	Up to 1/4 of the length of the leaf surface leaves were dead
5	The dead leaves were cup-shaped (U-shaped)	5	1/4–1/2 of the leaves were dead
7	The leaves were edge-bound (O-shaped)	7	More than 2/3 of leaves were dead
9	The leaves were completely curled	9	The whole plant was obviously dead

**Table 2** Summary of the locations and genetic effects of QTLs mapped using JL1/MY23 RILs under drought stress

Trait	Year	drought stress duration(D)	QTL	Chromosome	Genetic Interval(cM)	LOD	PVE(%)	Additive
LRI	2017	32	<i>qLRI1-1</i>	1	17.99–18.10	2.89	1.2	0.53
		32	<i>qLRI4-1</i>	4	39.20–40.32	3.82	1.63	-0.44
		32	<i>qLRI5-1</i>	5	0.00–1.28	12.82	5.85	0.84
		32	<i>qLRI5-2</i>	5	2.59–3.52	20.73	10.34	-1.12
		46	<i>qLRI7-1</i>	7	14.31–15.02	4.71	6.27	-0.49
		46	<i>qLRI7-2</i>	7	51.64–52.16	3.58	4.81	0.43
	2018	29	<i>qLRI2-1</i>	2	82.71–83.24	2.95	4.22	0.25
		29	<i>qLRI2-2</i>	2	103.75–104.50	9.69	12.75	0.44
		42	<i>qLRI2-2</i>	2	103.75–104.50	11.26	17.04	0.61
		57	<i>qLRI2-2</i>	2	103.75–104.50	5.68	10.59	0.42
		29	<i>qLRI3-1</i>	3	102.62–103.06	5.24	6.45	0.33
		57	<i>qLRI4-2</i>	4	2.57–3.77	2.66	4.79	0.28
		29	<i>qLRI4-3</i>	4	54.80–55.32	3.47	4.26	0.25
		57	<i>qLRI7-3</i>	7	45.34–46.41	5.92	11.07	0.43
		29	<i>qLRI8-1</i>	8	14.69–15.36	2.88	3.56	0.23
		29	<i>qLRI8-2</i>	8	66.90–67.02	4.49	5.68	0.29
		42	<i>qLRI12-1</i>	12	12.90–13.39	4.3	6.05	0.36
		2019	33	<i>qLRI1-2</i>	1	20.77–21.12	5.2	6.25
	33		<i>qLRI1-3</i>	1	135.79–136.44	2.81	3.3	0.21
	33		<i>qLRI2-3</i>	2	41.53–42.02	3.81	4.49	-0.24
	49		<i>qLRI5-3</i>	5	89.89–90.61	4.01	8.1	0.21
	63		<i>qLRI5-4</i>	5	93.85–94.46	6.3	11.13	0.41
	63		<i>qLRI12-1</i>	12	12.90–13.39	2.75	4.75	0.27
	49		<i>qLRI12-2</i>	12	13.39–14.06	3.67	7.32	0.2
33	<i>qLRI12-3</i>		12	14.06–15.71	5.26	6.48	0.3	
LWD	2017	32	<i>qLWD1-1</i>	1	55.92–56.51	6.36	9.37	-0.56
		46	<i>qLWD3-1</i>	3	147.66–148.39	2.54	3.62	0.34
		32	<i>qLWD4-1</i>	4	31.86–33.78	5.37	8.19	-0.52
		46	<i>qLWD4-1</i>	4	31.86–33.78	4.63	7.07	-0.48
		46	<i>qLWD5-1</i>	5	94.92–95.19	5.1	7.34	0.48
	2018	32	<i>qLWD5-2</i>	5	100.02–101.10	5.99	8.76	0.53
		57	<i>qLWD3-2</i>	3	140.04–141.48	2.73	4.77	0.2
		29	<i>qLWD4-2</i>	4	29.76–30.14	2.87	5.14	0.21
		42	<i>qLWD4-2</i>	4	29.76–30.14	3.29	5.26	0.24
		57	<i>qLWD4-2</i>	4	29.76–30.14	3.3	5.71	0.22
		57	<i>qLWD12-1</i>	12	14.06–15.71	3.56	6.77	0.24
	2019	63	<i>qLWD3-3</i>	3	70.52–71.14	3.66	4.86	-0.51
		49	<i>qLWD5-3</i>	5	89.89–90.61	3.07	6.15	0.3
		63	<i>qLWD5-4</i>	5	93.85–94.46	5.97	8.05	0.63
		33	<i>qLWD8-1</i>	8	11.59–12.30	2.66	5.76	-0.24
		63	<i>qLWD8-2</i>	8	89.04–90.63	3	3.95	0.46
	33	<i>qLWD9-1</i>	9	37.99–38.20	2.51	5.36	-0.23	
	63	<i>qLWD9-2</i>	9	70.83–71.61	3.34	4.47	0.47	
	49	<i>qLWD12-2</i>	12	13.39–14.06	4.16	8.4	0.36	

A positive additive effect indicates that the beneficial allele is derived from the male parent “Milyang 23”; a negative additive effect indicates that the beneficial allele is derived from the female parent “Jileng 1”

from the female parent JL1. In 2018, nine QTLs related to LRI were mapped to chromosomes 2, 3, 4, 7, 8, and 12. *qLRI2-2*, the major QTL, was repeatedly detected on days 29, 42, and 57 of drought treatment, with contribution rates of 10.59–17.04%. The beneficial alleles of all nine QTLs originated from the male parent. In 2019,

eight QTLs related to LRI were discovered on chromosomes 1, 2, 5, and 12. Among them, *qLRI5-4* explained the most variance (11.13%), and it was the major effect QTL. Three adjacent QTLs were mapped on chromosome 12, namely *qLRI12-1*, *qLRI12-2*, and *qLRI12-3*, with contribution rates of 4.75–7.32%. For all of these QTLs

except *qLRI2-3*, the allele increasing LRI was inherited from the male parent.

In 2017, five QTLs related to LWD were detected on chromosomes 1, 3, 4, and 5, explaining 3.62–9.37% of the phenotypic variation (Table 2). The additive effects of the three QTLs were positive, indicating that the beneficial alleles originating from the female parent. The additive effects of two QTLs were negative, indicating that the beneficial alleles originated from the male parent. *qLWD4-1*, located on chromosome 4, was detected on both day 32 and day 46 of drought stress, with contribution rates of 7.07% and 8.19%, respectively. In 2018, three QTLs related to LWD were identified on chromosomes 3, 4, and 12. Among these, *qLWD4-2* on chromosome 4 was detected on days 29, 42, and 57 of drought, with contribution rates ranging from 5.14 to 5.71%. The beneficial allele for this QTL came from the male parent. *qLWD12-1* was mapped to the same chromosome interval as *qLRI2-3*, which was detected in 2019. In 2019, eight QTLs associated with LWD were mapped to chromosomes 3, 5, 8, 9, and 12. These QTLs explained 3.95–8.40% of the observed phenotypic variation. The beneficial alleles of five of the QTLs were from the male parent.

During the tillering stage, 39 QTLs related to LRI and LWD were identified (Table 2). About three-fourths of these QTLs had confidence intervals less than 1 cM, with the smallest being 0.11 cM and the largest being 1.92 cM. The LOD scores ranged from 2.51 to 20.73, and the percent phenotypic variation ranged from 1.2 to 17.04%. Four QTLs, all of which were linked to LRI, explained more than 10% of the phenotypic variation. The positive alleles of the 25 QTLs were from the drought-tolerant parent MY23, while the positive alleles of 9 QTLs were derived from the drought-sensitive parent JLI. Overall, 23 QTLs related to LRI and 16 QTLs related to LWD were identified. Among these, *qLWD5-3* and *qLRI5-3*, *qLWD5-4* and *qLRI5-4*, *qLWD12-2* and *qLRI12-2*, and *qLWD12-1* and *qLRI12-3* were located in the same region of the same chromosome.

#### Discovery of Drought-Resistance Genes and Excellent Haplotypes in Rice

There were 281 genes located in the QTL region, with 201 genes located in the region with a LOD > 3. Among these genes, 147 were up-regulated and 54 were down-regulated. The most significantly up-regulated and down-regulated genes were located on chromosome 5. The gene with the highest up-regulated expression was located in a 0.00 cM–1.28 cM region with a LOD value of 12.82. Conversely, the gene with the most down-regulated expression was found in a 2.59 cM–3.52 cM region with a LOD value of 20.73. A total of 90 genes were found in the co-localized QTLs. Of these, 31 genes were repeatedly

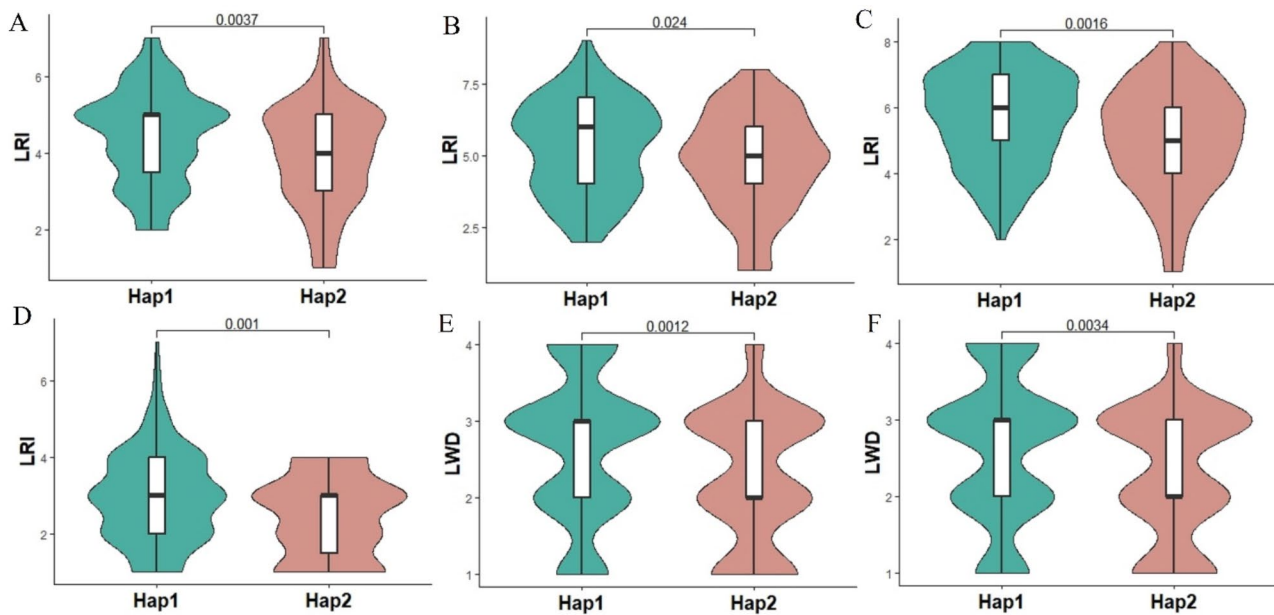
detected during the two-year drought tolerance identification study. Three drought resistance genes, *OsANN1*, *OsSLAC1*, and *OsTPS1*, are located on chromosomes 2 (31665723–31765723 bp), 4 (29020325–28882817 bp), and 5 (25576289–26094452 bp), respectively (Zang et al. 2011; Qiao et al. 2015; Sun et al. 2016).

During the haplotype analysis of the RIL population, two candidate genes with significant differences were noted in the intervals of 34,757,965–34,738,491 bp on chromosome 4 and 19,640,244–19,535,141 bp on chromosome 7. The sequences of non-homogeneous SNPs in the genes *Os04g0574600* (*LOC\_Os04g48520*) and *OsCHR731* (*LOC\_Os07g32730*) were extracted to identify these genes. These two genes were designated as candidate genes because of the presence of repeated allelic variants. The coding sequence of *Os04g0574600*, which is 2001 bp, contains four SNPs: 28,922,253 (T→C), 28,926,734 (C→T), 28,927,682 (G→T), and 28,928,513 (T→A). In the 1107 bp coding sequence of *OsCHR731*, there is one SNP (A→G). For both genes, there were two haplotypes, haplotype 1 (Hap1) and haplotype 2 (Hap2), present in the RIL population, there were significant differences in the phenotypic values of LRI and LWD between RILs carrying Hap1 and those carrying Hap2 ( $P < 0.05$ ,  $P < 0.01$ ) (Figs. 1 and 2). Specifically, the phenotypic values of LRI and LWD were much lower in Hap2-carrying RILs than in Hap1-carrying RILs. These results suggest that RILs carrying Hap2 of either *Os04g0574600* or *OsCHR731* exhibit stronger drought tolerance than those carrying Hap1.

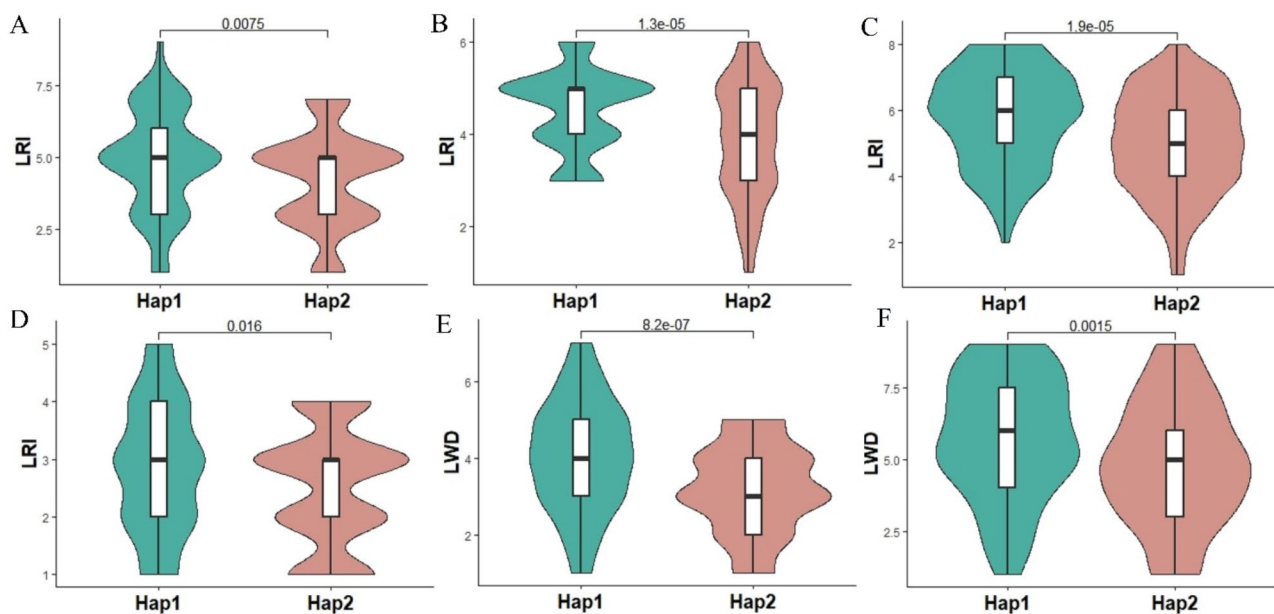
#### The Expression of Candidate Genes Related to Drought Tolerance Differs Between the Treatment Group and the Control Group

qRT-PCR analysis was performed to assess the difference in expression of candidate genes between the treatment and control groups. The expression levels of the candidate genes were generally higher in the treatment group than in the control group. Specifically, the expression of *Os04g0574600* was significantly higher in the treatment group than in the control group in five out of seven drought-tolerant samples tested. In the treatment group, the expression level of R8 was the highest (7.68 times that of the control group). R16 had an expression level 3.86 times higher than that the control group. R20 and R26 had expression levels 1.11 and 1.20 times higher than that of the control group, respectively, but these differences were not significant. In six out of the seven drought-tolerant samples tested, the expression of *OsCHR731* was considerably or extremely considerably higher than that of the control group. Like *Os04g0574600*, *OsCHR731* was most highly expressed in the R8 treatment group (19.47 times that of the control group). The expression level of *OsCHR731* in the R16 treatment group was 6.26





**Fig. 1** LRI and LWD of lines carrying one of two haplotypes of *Os04g0574600*. Drought stress treatment for 29 days in 2018 (A, E); drought stress treatment for 42 days in 2018 (B, F); drought stress treatment for 57 days in 2018 (C); drought stress treatment for 63 days in 2019 (D)



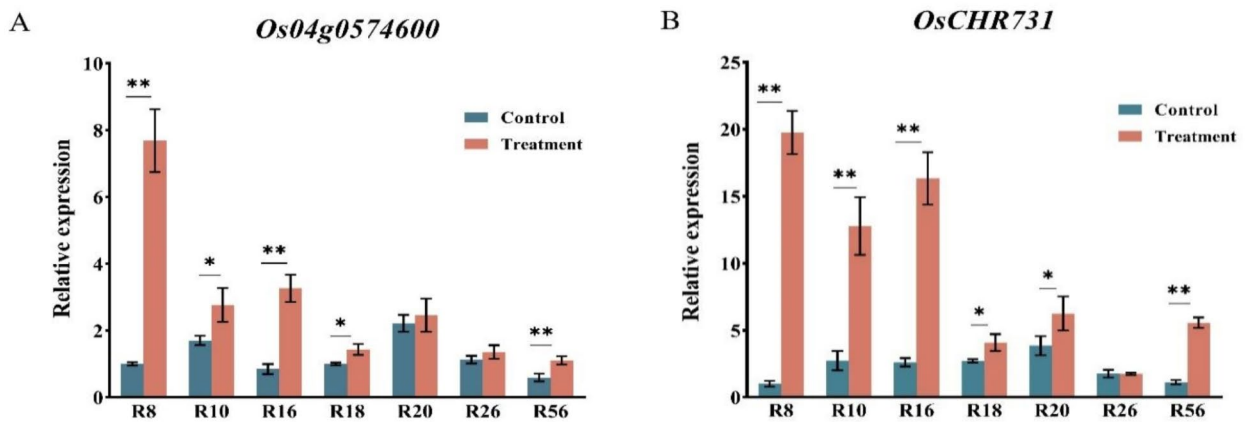
**Fig. 2** LRI and LWD of lines carrying one of two haplotypes of *OsCHR731*. Drought stress treatment for 46 days in 2017 (A); drought stress treatment for 29 days in 2018 (B); drought stress treatment for 57 days in 2018 (C); drought stress treatment for 63 days in 2019 (D, F); drought stress treatment for 49 days in 2019 (E)

times that of the control group. These results suggest that *Os04g0574600* and *OsCHR731* play an important part in regulating rice drought tolerance (Fig. 3).

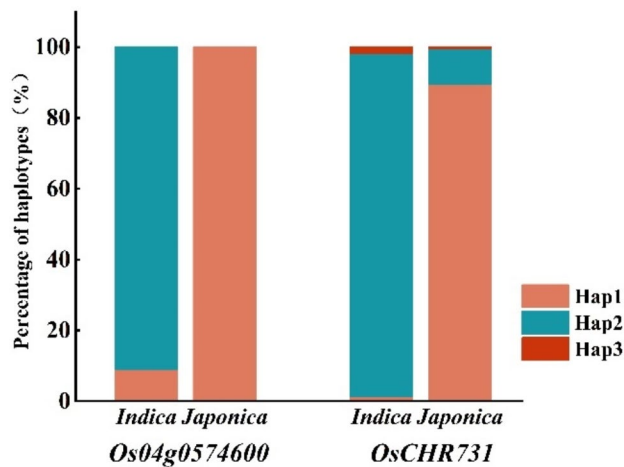
**Geographical Distribution of Haplotypes for Candidate Drought-Tolerance Genes**

To investigate the geographical distributions of the *Os04g0574600* and *OsCHR731* haplotypes, we analyzed

the haplotypes of 372 rice germplasm resources from 12 provinces in the Yangtze River and southern regions. The genomic data for these rice materials were obtained from the NCBI database (Liu et al. 2022). Of the rice germplasm resources, 209 were *indica* rice and 163 were *japonica* rice. There were two haplotypes (Hap1 and Hap2) of *Os04g0574600* in *indica* rice, accounting for 8.97% and 91.03% of accessions, respectively. In



**Fig. 3** Relative expression of candidate genes in drought-tolerant groups analyzed by qRT-PCR in both control and treatment conditions. Data are means  $\pm$  SD of three biological replicates. Significant differences were determined using two-tailed Student's t-test (\* $P < 0.05$ , \*\* $P < 0.01$ ). R is the family number



**Fig. 4** Distribution of candidate gene haplotypes in natural populations of *indica* and *japonica* rice

*japonica* rice, only Hap1 was detected. There were three haplotypes of *OsCHR731* in *indica* rice: Hap1, Hap2, and Hap3, accounting for 1.22%, 96.95%, and 1.83% of accessions, respectively. In *japonica* rice, 89.37%, 10.15%, and 0.48% of accessions had Hap1, Hap2, and Hap3, respectively (Fig. 4).

Haplotype analysis of rice germplasm resources showed that the proportion of accessions with the superior *Os04g0574600* haplotype Hap2 among the 12 provinces ranked as follows: Sichuan (93.33%)>Anhui (75.00%)>Yunnan (68.75%)>Fujian (66.67%)>Guangdong (64.71%)>Hunan (44.44%)>Jiangxi (37.50%)>Hubei (35.71%)>Guizhou (27.07%)>Jiangsu (25.00%)>Guangxi (23.53%)>Zhejiang (15.38%) (Fig. 5). In five of the provinces over 50% of accessions had Hap 2. The proportion of accessions with the superior haplotype of *OsCHR731*, Hap2, in 12 provinces ranked as follows: Sichuan (100%)>Yunnan (85.71%)>Anhui

(84.62%)>Fujian (80.00%)>Guangdong (77.78%)>Hubei (50.00%)>Guangxi (47.06%)>Hunan (44.44%)>Jiangxi (43.75%)>Guizhou (37.93%)>Jiangsu (26.67%)>Zhejiang (Fig. 6). In addition, Hap2 was found in more than 50% of accessions in five other provinces. Hap2 of both genes was dominant among the local rice resources of Sichuan, Yunnan, Anhui, Fujian, and Guangdong provinces, whereas a low proportion of the local rice resources of Zhejiang carried these haplotypes (Figs. 5A and 6A).

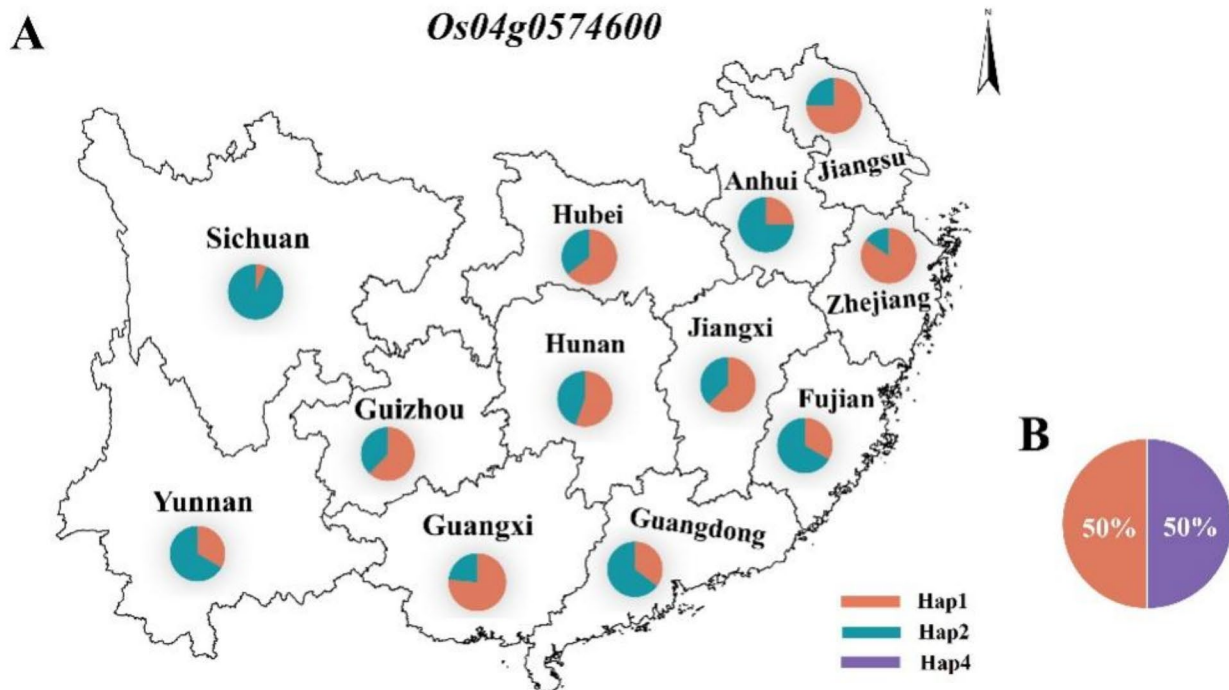
Haplotype analysis of wild rice resources showed that 50% carried Hap1 of *Os04g0574600* and 50% carried Hap4 (Fig. 5B), and 13.51%, 59.46%, and 27.03% carried Hap1, Hap2, and Hap3 of *OsCHR731*, respectively (Fig. 6B).

## Discussion

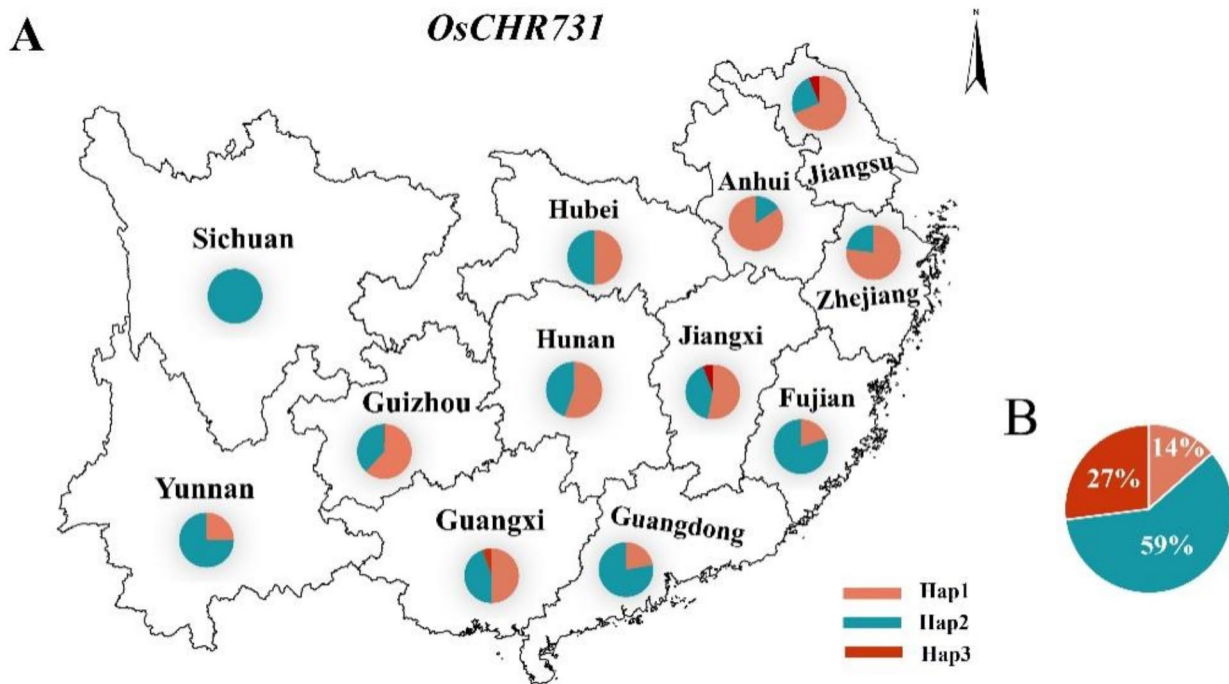
### Pleiotropic QTLs and Comparison with Previous Drought Tolerance QTLs

This study revealed that some QTLs may affect multiple traits owing to pleiotropy or tight linkage. For instance, *qLRI5-3* and *qLWD5-3*, *qLRI5-4* and *qLWD5-4*, *qLRI12-2* and *qLWD12-2*, and *qLRI12-3* and *qLWD12-1* were mapped to the same chromosome intervals and may control LRI and LWD simultaneously because of pleiotropy. All alleles were derived from the drought-tolerant parent MY23, and *qLRI5-4*, which controls LRI, explained the highest percentage of phenotypic variation (11.13%). Three QTLs, *qLRI12-1*, *qLRI12-2*, and *qLRI12-3*, on chromosome 12 are closely linked. This suggests that these genes may interact and have a cumulative effect, resulting in a synergistic enhancement of drought tolerance. These QTLs overlap with those reported in previous studies and may be hotspots for drought tolerance in rice.

In addition, 16 QTLs mapped in this study overlapped with previously reported chromosomal regions



**Fig. 5** Geographical distribution of the haplotypes of the candidate gene *Os04g0574600* in natural populations. **(A)** Distribution of candidate gene haplotypes in 12 provinces. **(B)** Distribution of candidate gene haplotypes in wild rice



**Fig. 6** Geographical distribution of haplotypes of the candidate gene *OsCHR731* in natural populations. **(A)** Distribution of candidate gene haplotypes in 12 provinces. **(B)** Distribution of candidate gene haplotypes in wild rice



(Table 3). *qLRI1-1* and *qLRI1-2*, located on chromosome 1, are in the same chromosomal region as *qLRI1-1<sup>J</sup>* and *qLWD1-2<sup>B</sup>*, which were mapped to an interval of 36,734,135–41,539,722 bp on chromosome 1 by Chen et al. (2023) using SSR markers. The additional nine QTLs were related to LRI and LWD under drought stress, as reported by Chen et al. (2023). Jiang et al. (2016) identified five QTLs related to drought tolerance. Three of these QTLs (*qSH-1*, *qRL-1-3*, and *qRS-1*) were associated with seedling height, maximum root length, and the ratio of root length to seedling height, and they are located in the same region on chromosome 1 and *qLRI1-1* and *qLRI1-2*. Ding (2011) identified QTL5 as being related

to the relative fertility rate under drought stress. In this study, *qLRI5-3*, *qLWD5-1*, and *qLWD5-3* were mapped to chromosome 5. The QTL intervals from our study are significantly narrower compared with those of previous studies, which improves the localization accuracy.

We compared the QTLs mapped using an ultra-high-density genetic linkage map with those mapped in the same population using SSR markers (Chen et al. 2023). The QTLs identified by Chen et al. (2023) were considerably different from those identified in this study. Chen et al. (2023) identified QTLs on all chromosomes except 8, 9, and 11, whereas we identified QTLs on chromosomes except 6, 10, and 11. This difference may be attributed to

**Table 3** QTLs for rice drought tolerance overlapping with previously reported QTLs

QTL	Chromosome	Location Interval	Previous research results			Trait
			Reference	Marker Interval	Location Interval	
<i>qLRI1-1</i>	1	39,830,874 – 39,682,995	Chen et al. (2023)	S01167A-RM315	36,734,135–41,539,722	LRI、LWD
			Jiang et al. (2016)	RM472-RM104	37,880,984–40,166,840	SH、RL、RS
<i>qLRI1-2</i>	1	38,642,574 – 38,534,609	Chen et al. (2023)	S01167A-RM315	36,734,135–41,539,722	LRI、LWD
			Jiang et al. (2016)	RM472-RM104	37,880,984–40,166,840	SH、RL、RS
<i>qLRI4-2</i>	4	34,757,965 – 34,738,491	Chen et al. (2023)	RM127-RM280	34,529,722–34,989,727	LRI
<i>qLRI7-1</i>	7	28,345,685 – 28,240,499	Zou (2007)	RM420-RM134	26,636,534–29,431,225	HD
<i>qLRI7-2</i>	7	19,640,244 – 19,535,141	Chen et al. (2023)	RM21657-RM21725	19,026,071–20,256,040	LRI
			Fu (2012)	RM11-RM18	19,256,914–25,652,511	BRT
<i>qLRI7-3</i>	7	20,730,769 – 20,628,398	Fu (2012)	RM11-RM18	19,256,914–25,652,511	BRT
<i>qLRI12-1</i>	12	25,747,684 – 25,637,712	Chen et al. (2023)	S12099-CMB1226.0	2,533,945–26,032,801	LRI、LWD
<i>qLWD1-1</i>	1	26,922,749 – 26,822,371	Chen et al. (2023)	RM259-RM522	40,670,655 – 5,792,766	LCC
<i>qLWD3-3</i>	3	17,533,795 – 17,422,997	Champoux MC et al. (1995)	RG482-RZ576	15,469,002–19,412,007	THK、R/S、DR/T
<i>qLWD4-2</i>	4	29,020,325 – 28,882,817	Zou (2007)	RM451-RM317	29,060,978 – 28,386,117	BY、PN、PH
<i>qLWD5-1</i>	5	26,237,907–26,308,755	Chen et al. (2023)	CMB0526.3-RM87	26,356,605–27,104,234	LRI、LWD
			Ding (2011)	RM274-RM421	23,976,333–26,848,154	MRDC、KGW
<i>qLWD5-2</i>	5	27,418,840–27,549,800	Teng (2002)	GA257-GA41	27,342,022–28,610,866	DT
<i>qLWD5-3/qLRI5-3</i>	5	24,942,036–25,030,149	Chen et al. (2023)	ID5010886-CMB0526.3	24,121,914–26,356,605	LRI、LWD
			Ding (2011)	RM274-RM421	23,976,333–26,848,154	MRDC、DIRD、DRVC
<i>qLWD5-4/qLRI5-4</i>	5	25,576,289–26,094,452	Chen et al. (2023)	ID5010886-CMB0526.3	24,121,914–26,356,605	LRI、LWD
			Yue et al. (2006)	RM274-RM421	23,976,333–26,848,154	MRDC、KGW
<i>qLWD12-1/qLRI12-3</i>	12	25,488,867 – 25,444,514	Chen et al. (2023)	S12099-CMB1226.0	2,533,945–26,032,801	LRI、LWD
<i>qLWD12-2/qLRI12-2</i>	12	25,637,712 – 25,488,867	Chen et al. (2023)	S12099-CMB1226.0	2,533,945–26,032,801	LRI、LWD

the variation in marker density and resolution. However, the large-effect QTLs were consistent, with both found on chromosomes 2 and 5. This study dramatically narrowed the marker interval and improved detection accuracy. In crop QTL studies, the lack of molecular markers is often a major limiting factor. Traditional molecular markers, such as RFLP, RAPD, and SSR markers, are often still far apart, which limits the resolution of QTL localization. The use of high-throughput sequencing technology has led to the establishment of high-density polymorphism markers, including SNPs and insertion-deletions, which increases the accuracy of QTL localization (Geng et al. 2023).

We constructed a high-density genetic map using whole-genome resequencing data. The map had a marker density of 7.83 markers/Mb with an average interval of 0.48 cM between adjacent bin markers (Geng et al. 2023). The density of the markers used by Chen et al. was 1.28 markers/Mb with an average interval of 10.12 cM between adjacent markers (2023). The significantly higher marker density on our map, resulted in a reduction of the confidence intervals of QTLs and an improvement in the resolution of QTL localization. For instance, *qLRIS-4* and *qLWD5-4*, which control LRI and LWD, were mapped to a 518.16 kb interval (25576289–26094452 bp) on chromosome 5. For comparison, the same QTL mapped using SSR markers was located in a 2.23 Mb interval (24121914–26356605 bp) on chromosome 5 (Chen et al. 2023). Similarly, *qLWD5-1*, which controls LWD, was mapped to a 70.88 kb interval (chr05: 26237907–26308755 bp) and explained 7.34% of the phenotypic variance, whereas using SSR markers, this QTL was mapped to a 747.63 kb interval (chr05: 26356605–27104234 bp) and explained 5.25% of the phenotypic variance. The high-density genetic map substantially narrowed the range of QTL intervals, allowing for more accurate location of the functional gene. QTL fine-mapping results not only increase the probability of finding functional genes, but also reflect the great advantage of performing high-density QTL mapping.

#### Validation of Candidate Genes for Drought Tolerance

Rice drought tolerance is quantitative trait controlled by multiple genes and a complex genetic mechanism. The rapid development of next-generation sequencing technology has accelerated QTL mapping and gene discovery in rice. These QTLs contain a large number of functional genes. Although researchers have used various techniques to mine drought-tolerance genes, only a few genes showing significant effects have been thoroughly studied or cloned. Most of these genes were identified as being differentially expressed under drought stress conditions, and their functions were determined by reverse genetics. In this study, we cloned three drought-tolerant

genes identified in the QTL regions on chromosomes 2, 4, and 5, demonstrating that resequencing technology and linkage analysis are efficient means to identify rice drought-tolerance genes. In addition, the effectiveness of this method in mining rice salt tolerance genes has also been demonstrated (Geng et al. 2023).

Of the 281 identified genes, two candidate genes, *Os04g0574600* and *OsCHR731*, were selected based on gene function annotation, and their gene expression levels were analyzed by qRT-PCR. The expression levels of the two genes under drought stress were significantly higher than those under normal growth conditions, indicating their close association with drought tolerance. The candidate gene *Os04g0574600* is located in *qLWD4-2* on chromosome 4, which has frequently been detected and is located within the QTL interval identified by Zou et al. (2007). The gene *OsCHR731* is located at the *qLRI7-2* locus on chromosome 7 and has been recognized as a QTL by Fu et al. (2012) and Chen et al. (2023). In a previous study, qRT-PCR analysis of 14-day-old subjected to drought stress revealed that *OsCHR731* was up-regulated after 12 h of treatment (Guo et al. 2022), which is consistent with our findings.

*Os04g0574600* encodes a ZOS4-12-C2H2 zinc finger protein. C2H2 zinc finger proteins play an essential role in various developmental processes in plants, such as regulating plant growth, hormone signaling, and responses to biotic and abiotic stresses (Zhang et al. 2016). Several homologous genes of *Os04g0574600* have been closely linked to drought resistance in rice, Arabidopsis, and tomato (Xu et al. 2008; Luo et al. 2012; Li et al. 2018; Feng et al. 2023). *OsCHR731* encodes a Snf2 family protein. Many rice Snf2 genes exhibit tissue-specific expression patterns, and some of these genes are highly responsive to abiotic stresses, such as drought, salt, and low temperature (Guo et al. 2014, 2022). Based on these results, we speculate that *Os04g0574600* and *OsCHR731* may be closely related to drought tolerance in rice, and are worthy of further study.

#### Haplotype Analysis and Geographical Distribution of Candidate Genes

Even though whole-genome sequencing technology can provide comprehensive genetic information at the whole-genome level, it cannot distinguish allelic differences on homologous chromosomes. In contrast, haplotype analysis can more deeply analyze the combination of different genomic loci and their genetic characteristics in a single chromosome or a specific chromosome region. This is particularly important for the study of heterosis and the discovery of finer allelic variation (Ma et al. 2017). Haplotype analysis can reveal the genetic background of a trait by identifying the genetic markers associated with that trait. It can also provide more information on

allelic diversity and improve genotyping accuracy (Lu et al. 2012). Haplotype analysis of the rice grain width gene *GS5*, rice pre-harvest sprouting gene *Os04g0545200*, and salt tolerance genes (*SKC1/OsHKT8*, *qSE3/OsHAK21*, and *RST1/OsARF18*) were analyzed by researchers (Yi et al. 2016; Min et al. 2023; Li et al. 2023) revealed haplotypes contributing to good trait values. The discovery of superior haplotypes such as these provides molecular markers for effective screening of genes related to high yield and stress resistance. It also offers significant scientific and technological support for rice breeding.

Haplotype analysis has been also been used to identify natural variants and beneficial alleles associated with drought tolerance, such as *DROT1*, in rice germplasm resources. Hap1 and Hap4 are haplotypes of *DROT1* primarily found in *japonica* and *indica* rice, respectively. In upland rice, Hap3 is present at a much higher frequency than other haplotypes. A key SNP (C→T) in the promoter region dramatically enhances the expression of *DROT1* in upland rice, thereby improving drought tolerance (Sun et al. 2022). Here, we performed haplotype analysis of 132 genes within the QTL interval. SNPs in *Os04g0574600* and *OsCHR731* were found in both the RIL population and rice germplasm resources. In the RIL population, the materials carrying Hap2 of *Os04g0574600* or Hap2 of *OsCHR731* showed strong drought tolerance. This indicates that these haplotypes have enormous potential for utilization in rice breeding. Hap2 of *Os04g0574600* was found exclusively in local *indica* rice germplasm resources. However, Hap2 of *OsCHR731* was found in significantly more *indica* rice accessions than *japonica* rice accessions, suggesting that this haplotype could be effectively utilized in *japonica* rice breeding.

The distribution of haplotypes (Figs. 5 and 6) indicates that the proportion of rice germplasm resources carrying the superior haplotypes (Hap2) of *Os04g0574600* and *OsCHR731* is higher in Sichuan, Yunnan, Anhui, Fujian, and Guangdong compared with other provinces. This distribution pattern may reflect the selection pressure exerted by specific climatic and soil conditions, as well as agricultural activities; specifically, the superior haplotypes of *Os04g0574600* and *OsCHR731* were selected for in local rice germplasm resources. The comparison of haplotypes between wild rice and native rice varieties revealed that the former contained Hap1 and Hap4 of *Os04g0574600*, whereas the latter contained Hap1 and Hap2. During rice evolution, Hap1 of the *Os04g0574600* adapted to environmental stresses, such as drought stress, and anthropogenic selective pressures, and was retained, while Hap4 was eliminated. The wild rice accessions contained three haplotypes of *OsCHR731*: Hap1, Hap2, and Hap3. While Hap1 and Hap2 were present in local rice species, Hap3 was only found in a few provinces and was rare. It is important to emphasize that Hap3 was found

in a very low proportion of rice accessions. It is possible that Hap1 and Hap2 have been conserved in rice because of their potent environmental adaptability, while Hap3, lacking such adaptability, has become a rare haplotype.

## Conclusion

Thirty-nine QTLs related to drought tolerance were identified and mapped to nine chromosomes. A novel gene, *Os04g0574600*, which is closely associated with rice drought tolerance, was discovered. The expression levels of this gene under drought stress were much higher than those under normal conditions, indicating that it functions as a positive regulator. In the RIL population, Hap2 of this gene is closely associated with intense drought tolerance; thus, it is an excellent haplotype. In Sichuan, Yunnan, Anhui, Guangdong, and Fujian provinces, the proportion of indigenous rice varieties carrying the Hap2 haplotype was significantly higher than that in other provinces. The proportion of *indica* rice accessions with Hap2 is much higher than that of *japonica* rice accessions; thus, Hap2 has greater potential for utilization in future *japonica* rice drought tolerance breeding.

## Abbreviations

QTL	Quantitative trait locus
RIL	Recombinant Inbred Line
LRI	Leaf Rolling Index
LWD	Leaf Withering Degree
LCC	Leaf Chlorophyll Content
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
JL1	Jileng 1
MY23	Milyang 23
SSR	Simple Sequence Repeat
MAS	Marker-Assisted Selection
SSD	Single Seed Descent
LOD	Logarithm of the Odds
PVE	Phenotypic Variation Explained
VCF	Variant Call Format
ANOVA	Analysis of Variance
NCBI	National Center for Biotechnology Information
RFLP	Restriction Fragment Length Polymorphism
RAPD	Random Amplified Polymorphic DNA

## Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12284-024-00733-9>.

Supplementary Material 1

## Author Contributions

AJ conducted the data analysis and wrote the manuscript. LC and JM carried out the field work and generated the phenotypic data. BH provided assistance with the field work. XM and DC were responsible for collecting the genotype data. JS and LH designed the experiment, guided the experiments, and revised the manuscript. All authors have read and approved the final version of the manuscript.

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Technology Innovation Program, and the National Crop Germplasm Resources Center (NCGRC-2023-02).

#### Data Availability

No datasets were generated or analysed during the current study.

#### Declarations

#### Ethical Approval

This study complied with the ethical standards of China where this research was performed.

#### Consent for Publication

Not applicable.

#### Competing Interests

The authors declare no competing interests.

#### Author details

<sup>1</sup>Institute of Crop Germplasm Resources, Guizhou Academy of Agricultural Sciences, Guiyang 550006, China

<sup>2</sup>Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China

<sup>3</sup>State Key Laboratory of Crop Gene Resources and Breeding, Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China

<sup>4</sup>Institute of Crop Science, Ningxia Academy of Agricultural and Forestry Science, Yinchuan 750001, China

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