

WDR6K, a designed SNP array for the research and improvement of rice drought-resistance

Ziming Li ^{a,c,1}, Lei Wang ^{c,d,1}, Yi Liu ^{a,c,d,1}, Xiaosong Ma ^{c,d},
Anning Zhang ^{c,d}, Zhi Luo ^c, Ming Yan ^{c,d}, Ligu Zhou ^{c,d}, Liang Chen ^{c,d},
Lijun Luo ^{a,b,c,d,*}, Hui Xia ^{a,b,c,d,**}

^a College of Plant Sciences & Technology, Huazhong Agricultural University, Wuhan 430070, China

^b College of Agriculture, South China Agricultural University, Guangzhou 510642, China

^c Key Laboratory of Grain Crop Genetic Resources Evaluation and Utilization, Ministry of Agriculture and Rural Affairs, Shanghai Agrobiological Gene Center, Shanghai 201106, China

^d National Key Laboratory of Crop Genetic Improvement and National Center of Plant Gene Research, Hubei Hongshan Laboratory, Huazhong Agricultural University, Wuhan 430070, China

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ABSTRACT

Water-saving and drought-resistance rice (WDR) integrates many green characters (e.g., drought resistance, water/nutrient use efficiency, direct seeding, etc.) from upland rice into the elite paddy rice. The molecular designed breeding is a promising breeding technology for WDR based on the high-throughput genotyping. Therefore, an efficient and low-cost genotyping tool for WDR breeding, which is suitable in the foreground selection of drought resistance and other green traits, is required. In this study, we designed a SNP array named as WDR6K with special strategies in loci selection. WDR6K contains 5895 SNP/InDel loci distributes evenly across the rice genome. Compared with other rice 6K SNP arrays, WDR6K possesses a high proportion (>90.0 %) of missense SNPs without apparent loss of polymorphism. Moreover, WDR6K possesses considerable amount (~41.0 %) of upland-lowland differentiated loci, by which we can identify and utilize genetic resources of upland rice. WDR6K refers to 5001 genes, including hundreds of known genes of green characters required by WDR. These genes are related to rice drought resistance as indicated by the GO/KEGG enrichments and their greater expression responses to drought, osmotic, and oxidative stresses and abscisic acid. Furthermore, WDR6K shall be closely associated with drought resistance as it contains 922 drought resistance contributing loci, which can facilitate the foreground selection for drought resistance. Finally, developed by the liquid phase genotyping by pinpoint sequencing of captured targets technology, WDR6K is low-cost and flexible for further version updates to add markers. Therefore, WDR6K has great prospective in the molecular designed breeding of WDR.

Introduction

The crop breeding has entered in a genomic era with the fast development of the high-throughput genotyping and phenotyping technologies since the last decade (Hickey et al., 2019). Molecular designed breeding is a promising strategy to replace conventional breeding methods, particularly for complicated traits (Zuo and Li, 2014). Molecular designed breeding requires an efficient genotyping tool for breeders to apply the precise foreground selection, as well as the

background selection (Singh et al., 2015). The foreground selection is to select beneficial alleles for the target traits based on the knowledge of associations between traits and molecular markers (Alemu et al., 2024). The background selection is to select breeding lines with high similarities to the recurrent parent based on genome-wide markers (Karunaratne et al., 2021). Therefore, a well-designed system of molecular markers for breeders requires a low- to medium-resolution genomic markers to assist the background selection, while a set of traits-/function- associated loci for the foreground selection (Hasan

* Corresponding author at: College of Plant Sciences & Technology, Huazhong Agricultural University, Wuhan 430070, China.

** Corresponding author at: College of Agriculture, South China Agricultural University, Guangzhou 510642, China.

E-mail addresses: lijun@sagc.org.cn (L. Luo), hxia@sagc.org.cn (H. Xia).

¹ Authors contributed equally to this work.

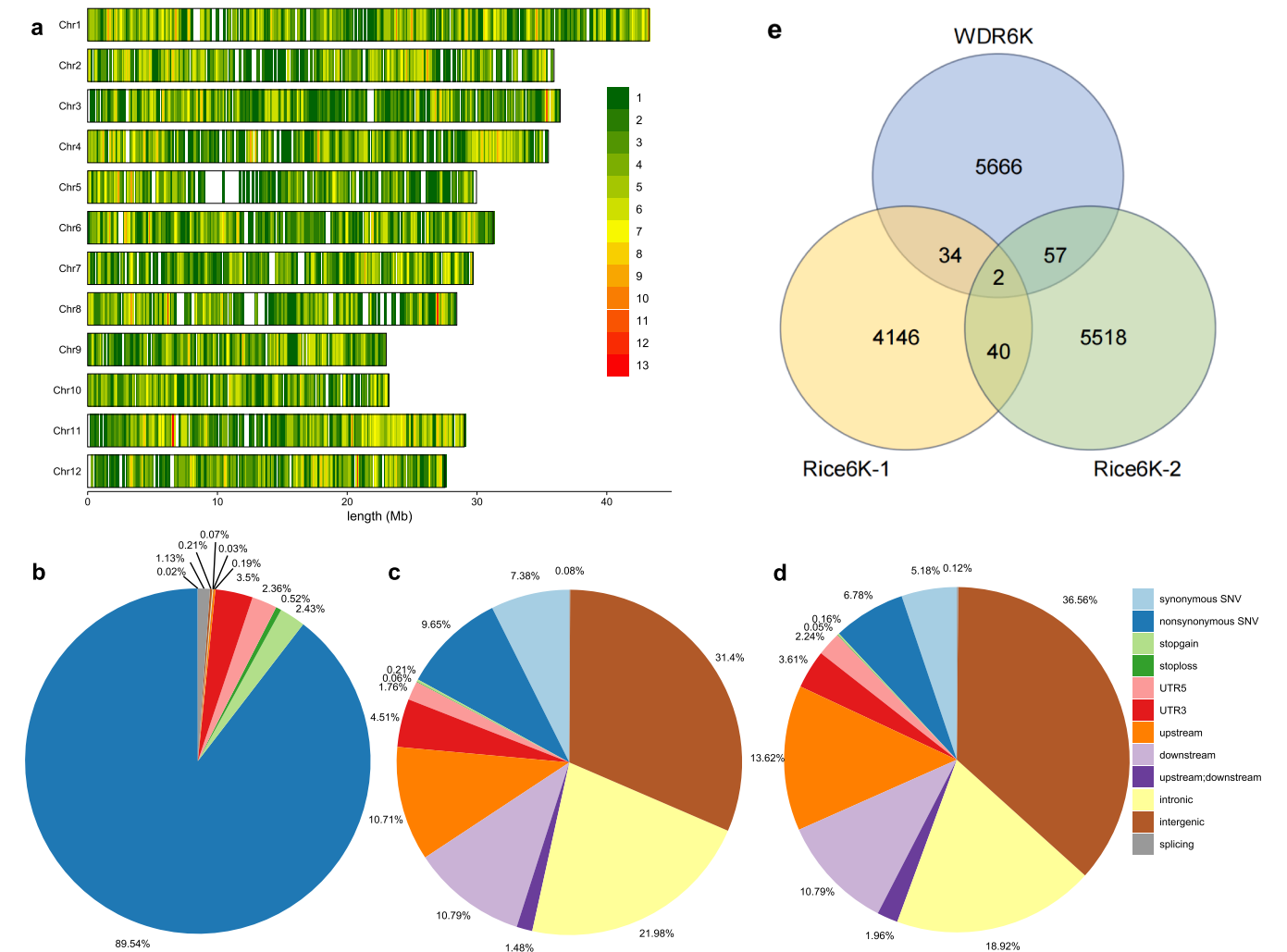


Fig. 1. Information of selected markers in WDR6K. **a.** Genomic distribution of 5895 selected markers in WDR6K. **b.** Percentages of different types of SNP loci in by WDR6K. **c.** Percentages of different types of SNP loci in by Rice6K-1. **d.** Percentages of different types of SNP loci in by Rice6K-2. **e.** Venn diagram of selected SNPs of WDR6K, Rice6K-1, and Rice6K-2.

Table 1
Basic information of the genomic distribution of SNPs in WDR6K.

Chromosome	No. of SNPs	No. of covered genes	Maximum gap length (bp)	Average gap length(bp)
Chr1	763	649	701,015	56,691.2
Chr2	552	486	582,917	64,875.3
Chr3	546	498	751,116	66,272.1
Chr4	576	494	502,531	61,491.3
Chr5	353	315	1,446,896	84,214.5
Chr6	513	431	611,317	60,653.9
Chr7	462	380	861,602	64,231.6
Chr8	418	358	945,003	67,724.5
Chr9	366	319	456,166	62,587.6
Chr10	376	331	411,897	61,665.3
Chr11	524	438	668,600	54,969.8
Chr12	446	377	504,990	61,097.8
Overall	5895	5076	1,446,896	63,042.3

et al., 2021). In addition, given the large amounts of tests during the breeding procedure, the SNP array should be economic efficient.

Among various molecular markers, single nucleotide polymorphisms (SNPs) are the most powerful and efficient markers in the high throughput genotyping in molecular breeding (Guo et al., 2021). Compared with genotyping by sequencing, it is easier in data collection and data analysis (Singh et al., 2015). Therefore, the SNP array is a good

Table 2
Percentage of array SNPs in different gradients of allele frequency among the source population and the RICE3K population.

Frequency gradients of the reference allele	Percentage of SNPs according to the source population			Percentage of SNPs according to RICE3K		
	WDR6K	Rice6K-1	Rice6K-2	WDR6K	Rice6K-1	Rice6K-2
≥ 0.9	6.25 %	18.69 %	1.01 %	12.57 %	12.62 %	1.26 %
$0.9 > x \geq 0.8$	32.78 %	18.45 %	18.27 %	9.36 %	12.72 %	4.45 %
$0.8 > x \geq 0.7$	25.33 %	21.46 %	38.13 %	13.41 %	9.73 %	7.44 %
$0.7 > x \geq 0.6$	9.31 %	18.69 %	28.74 %	13.32 %	8.03 %	9.71 %
$0.6 > x \geq 0.5$	8.14 %	7.98 %	8.86 %	12.33 %	8.34 %	13.73 %
$0.5 > x \geq 0.4$	8.30 %	4.69 %	3.31 %	10.35 %	9.38 %	18.25 %
$0.4 > x \geq 0.3$	6.53 %	3.60 %	1.08 %	7.29 %	16.65 %	29.66 %
$0.3 > x \geq 0.2$	2.55 %	2.75 %	0.41 %	7.00 %	9.52 %	10.28 %
$0.2 > x \geq 0.1$	0.73 %	2.58 %	0.14 %	8.98 %	5.97 %	4.48 %
$0.1 > x \geq 0$	0.07 %	1.11 %	0.05 %	5.40 %	7.03 %	0.73 %

Table 3

Percentage of genomic and array SNPs in different gradients of allele frequency (AF) differences between typical upland (DTU) and lowland rice (DTL).

Gradients of AF differences between DTU and DTL	Percentage of SNPs in different gradients			
	Genomic	WDR6K	Rice6K-1	Rice6K-2
≥ 0.9	0.09 %	0.87 %	0.24 %	0.09 %
$0.9 > x \geq 0.8$	0.40 %	3.35 %	0.92 %	0.28 %
$0.8 > x \geq 0.7$	0.82 %	5.82 %	1.80 %	0.64 %
$0.7 > x \geq 0.6$	1.42 %	7.84 %	2.56 %	1.49 %
$0.6 > x \geq 0.5$	1.88 %	8.26 %	3.39 %	2.04 %
$0.5 > x \geq 0.4$	2.17 %	8.31 %	4.41 %	2.50 %
$0.4 > x \geq 0.3$	2.27 %	6.53 %	4.26 %	2.02 %
$0.3 > x \geq 0.2$	3.30 %	7.23 %	5.12 %	3.70 %
$0.2 > x \geq 0.1$	6.40 %	3.89 %	8.72 %	5.83 %
$0.1 > x \geq 0$	81.25 %	47.90 %	68.59 %	81.40 %

choice in molecular designed breeding. Several medium- or high-resolution SNP arrays have been developed for genetic study and breeding in rice, including two 6K arrays (Yu et al., 2014; Thomson et al., 2017), a 44K array (Zhao et al., 2011), two 50K (Singh et al., 2015; Chen et al., 2014), and a 700K high-density rice array (McCouch et al., 2016). These above-mentioned SNP arrays are mainly designed for and well applied in the genetic study and breeding for agronomy important traits determined by main effect QTLs, such as the yield and quality.

The improvement of drought resistance is an important goal in rice breeding (Yu et al., 2021), particularly given the frequently occurred

water-deficiency and drought world-wide due to the global climate change (Pokhrel et al., 2021). The molecular designed breeding for drought resistance is necessary and ongoing. Unfortunately, drought resistance is a complex quantitative trait compromised by several mechanisms and an elaborate regulatory network with hundreds of genes (Hu et al., 2023). Till the end of 2023, >300 genes have been reported to be associated with rice drought resistance. However, most drought resistant genes have been functionally characterized by over-expression, knockout, and/or knock down transgenic plants. As a result, we know little about the beneficial allele and allelic contribution of a gene to drought resistance, which greatly limits the selection of foreground markers in breeding.

Upland rice possesses abundant genetic diversities in terms of green characters (e.g., drought resistance, direct seeding, tolerance to nutrient deficiency, etc.) due to its adaptation to upland dry cropping ecosystem (Xia et al., 2019; Luo et al., 2022). Due to the bi-directional selection between drought resistance and productivity, upland rice thus retains considerable genetic diversities on drought resistance (Xia et al., 2019). Many beneficial alleles of drought resistant genes have been identified in upland rice (Xia et al., 2014; Sun et al., 2022; Li et al., 2023; Wang et al., 2024). As a result, upland-specific or dominant alleles should be good candidates in the foreground selection for drought resistance and other green characters (Luo et al., 2022). However, most source populations from which SNP loci are extracted and designed for the previous SNP arrays, have limited upland genotypes. This may cause the exclusion of upland-lowland differentiated SNP loci due to their low frequencies. The lack of upland-specific or dominant alleles from the

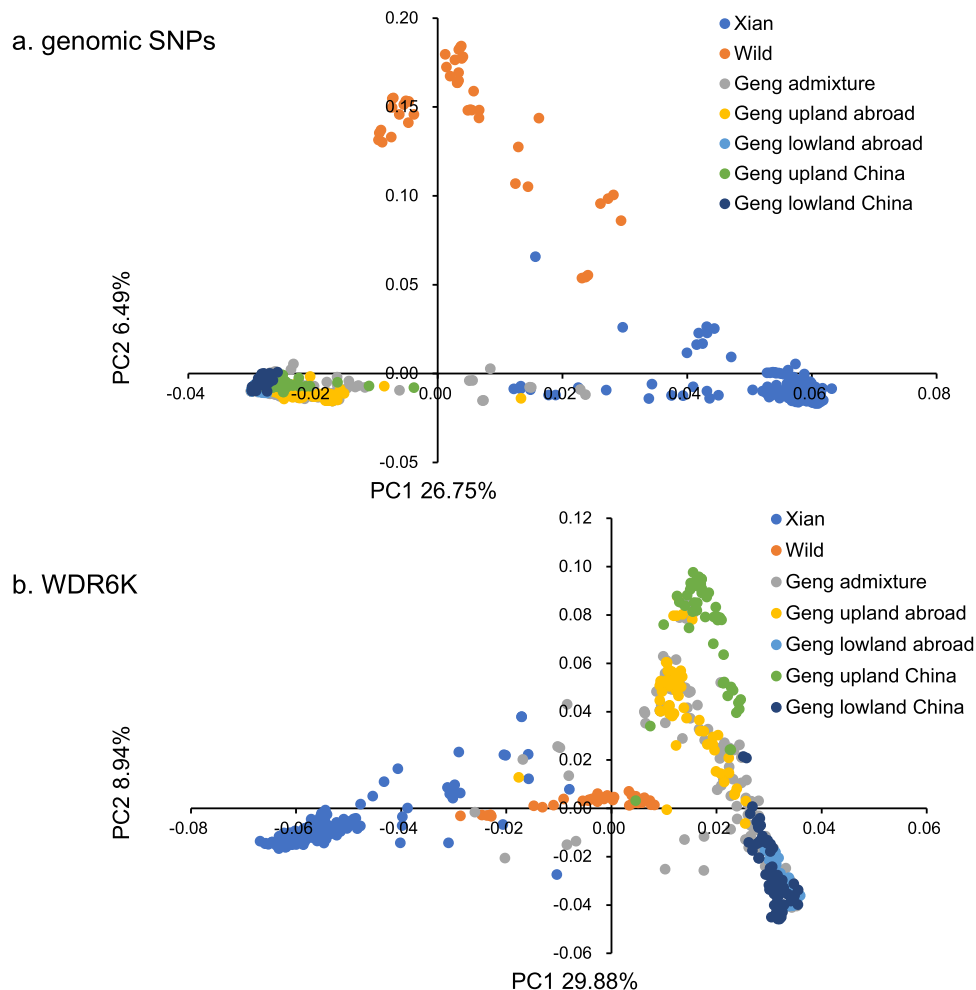


Fig. 2. Results of principal component analysis among the source population based on genomic SNPs (a) and selected SNPs in WDR6K (b). The source population, where SNPs of WDR6K are generated, has 762 rice genotypes with typical upland and lowland rice (Table S1).

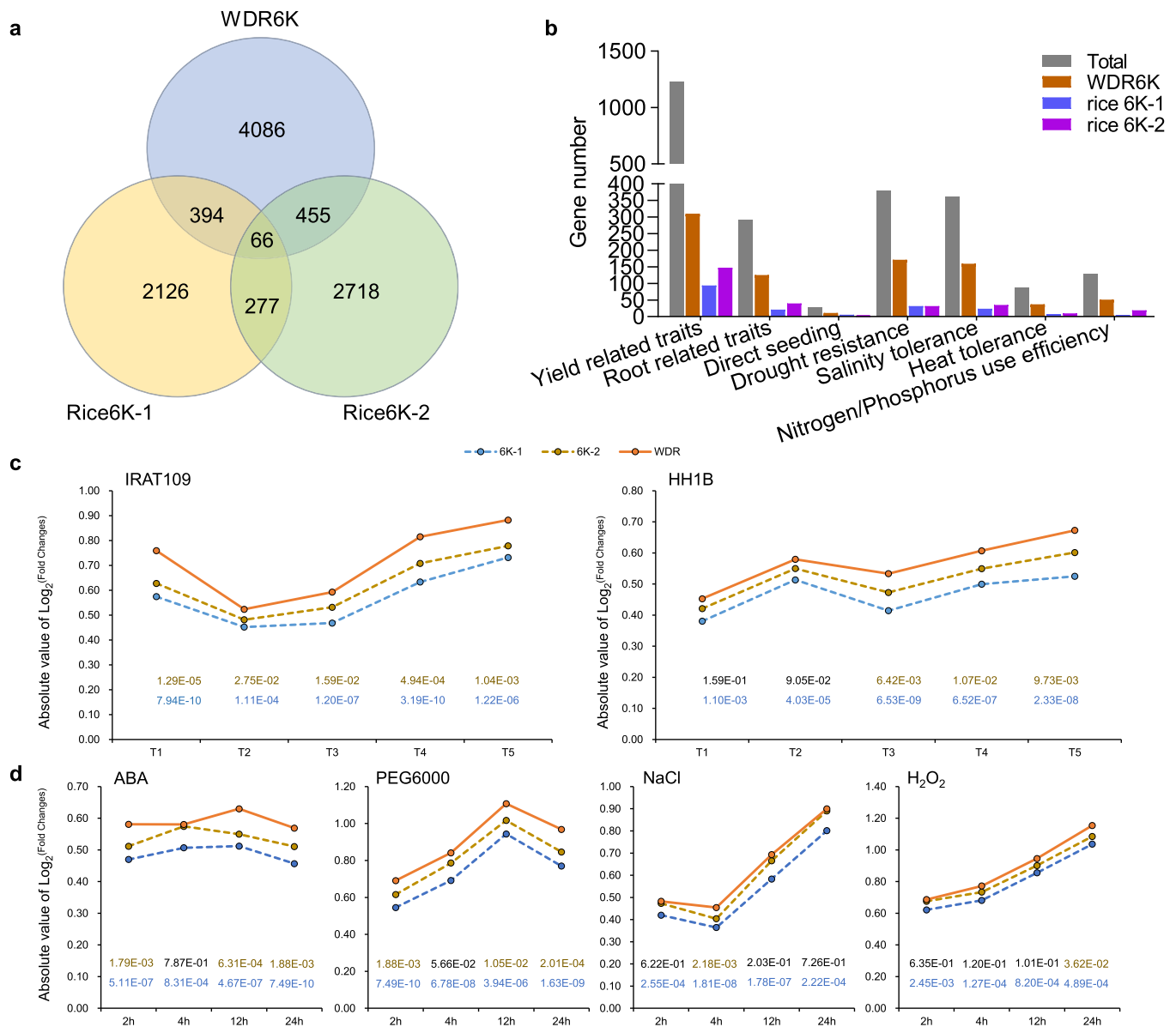


Fig. 3. Comparisons of genes covered by WDR6K, Rice6K-1, and Rice6K-2. **a.** Venn diagram of genes covered by WDR6K, Rice6K-1, and Rice6K-2. **b.** Number of known genes associated with different functions covered by WDR6K, Rice6K-1, and Rice6K-2. **c.** Averaged fold changes of genes covered by WDR6K, Rice6K-1, and Rice6K-2 in response to the progressive field drought in two rice genotypes (IRAT109 and HH1B). **d.** Averaged fold changes of genes covered by WDR6K, Rice6K-1, and Rice6K-2 in response to treatments of 100 μmol/L abscisic acid (ABA), 15 % PEG6000, 150 mmol NaCl, and 1 % H₂O₂ in Nipponbare. The *p* values in blue and brown indicate significant differences between WDR6K and Rice6K-1 and those between WDR6K and Rice6K-2 by the independent *t*-test, respectively.

ecotype-differentiated loci shall greatly block the utilization of upland green genetic resources in breeding.

The WDR (NY/T2862–2015) is commonly bred from the crossing of elite paddy rice and upland rice. It combines the both advantages from the elite paddy rice (high yield and good quality) and upland rice (water-saving characters and drought resistance) (Luo, 2010; Luo et al., 2019). WDR aims to be planted in the low-to-medium-yield field with poor irrigation conditions by the resource-saving (e.g., direct seeding, less fertilizer, and non-irrigation) cultivation (Luo et al., 2019; Xia et al., 2022). It thus requires many green characters that retained in upland rice such as drought resistance, high water and nutrient use efficiency, tolerances to direct seeding and some other abiotic stresses accompanied with drought (e.g., heat stress, soil-salinity, etc.). The core genetic basis of WDR breeding is to relive the upland green genetic resource in the genetic background of elite rice (Luo et al., 2019). Therefore, it is necessary to develop a SNP array specific to WDR breeding with

sufficient upland-specific or dominant alleles.

In this study, we designed a 6K SNP array (named WDR6K ver. 1.0) with special strategy of SNP loci selection that differs from previous designs of rice SNP arrays, from which great number of upland-lowland differentiated loci were included. We also provided some evidences that the selected SNP loci in WDR6K were closely associated with drought resistance and many other green characters required by WDR. Therefore, we can apply the foreground selection precisely with the developing knowledge of beneficial alleles of drought resistance. Furthermore, the application of the liquid phase genotyping by pinpoint sequencing of captured targets (cGPS) technology greatly reduce the cost of genotyping by this array. The cGPS technology can also provide the flexibility for further updates of SNP loci following the advances of functional genomics in rice drought resistance. It represents great prospective in the improvement of drought resistance in the coming molecular designed breeding of WDR.

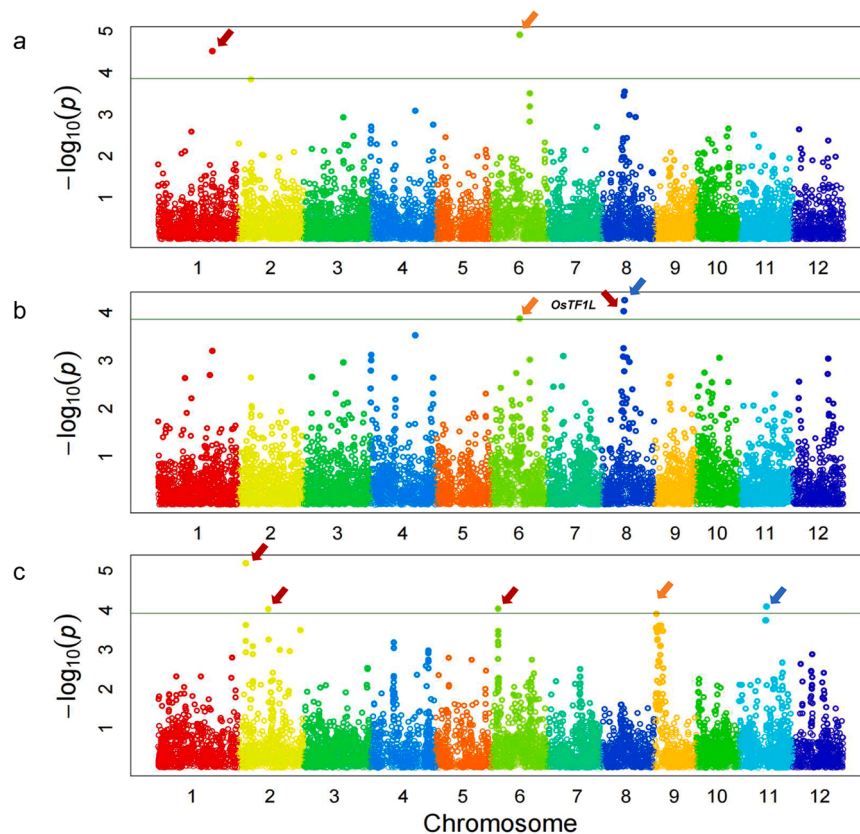


Fig. 4. Manhattan plots of results from genome-wide association study (GWAS) for drought resistance based on combined SNPs from WDR6K (indicated by red arrows), Rice6K-1 (indicated by blue arrows), and Rice6K-2 (indicated by yellow arrows). **a.** Manhattan plot of GWAS for the drought resistance quantified by drought resistant coefficient evaluated in 2011. **b.** Manhattan plot of GWAS for the drought resistance quantified by drought resistant coefficient evaluated in 2012. **c.** Manhattan plot of GWAS for the drought resistance quantified by relative grain weight evaluated in 2016.

Materials and methods

SNP source for the array design

In previous studies, we have created a source population with 762 rice genotypes world-wide (Table S1) for the genetic dissection of rice drought resistance and studying the genetic differentiation between upland and lowland rice ecotypes (Xia et al., 2019; Luo et al., 2022). This source population can be divided into seven groups as: Wild, *Xian*, *Geng* admixture, *Geng* upland abroad, *Geng* lowland abroad, *Geng* lowland China (also defined as typical lowland rice), and *Geng* upland China (also defined as typical upland rice). As indicated by the previous findings that upland rice possesses abundant genetic diversity of many green characters (e.g., drought resistance, nutrient use efficiency, direct seeding, etc.) (Luo et al., 2022), the considerable proportion of upland rice landraces involved in this population ensures the presence of upland-specific/dominant alleles in the SNP array. All these rice genotypes have been re-sequenced and >6000,000 SNPs have been totally identified. The raw data have been deposited into the NCBI Sequence Read Archive (SRA) under the accession number PRJNA260762 and the procedures for SNP calling and have been described in Luo et al. (2022).

Design of the WDR6K (ver. 1.0)

The design of an SNP array for WDR breeding has four considerations: (1) Background selection which requires sufficient markers that are evenly distributed across the rice genome. (2) Widely applicable within *Xian* (*indica*), *Geng* (*japonica*), and inter-subspecies breeding. (3) Foreground selection for green characters (e.g., drought resistance, tolerances to heat and salinity, high nutrient efficient, etc.) required by

WDR. (4) Foreground selection for other agronomic important traits.

For this purpose, we select SNP loci for this array following the principles below:

- (1) Dividing the rice genome into 200K windows. Six SNP loci were designed for each window to involve adequate evenly distributed markers.
- (2) Two SNP loci were designed for each 200K-window to represent the genetic diversity within *Xian* (*indica*) subspecies, whose allele (ref or alt) frequencies (AF) were between 0.10 and 0.90 in this subspecies.
- (3) Two SNP loci were designed for 200K-window to represent the genetic diversity within *Geng* (*japonica*) subspecies, whose allele (ref or alt) frequencies (AF) were between 0.10 and 0.90 in this subspecies.
- (4) Two SNP loci were designed for 200K-window to separate subspecies, whose differences in AF between *Xian* and *Geng* were >0.30.
- (5) Non-synonymous SNPs, particularly those located in function-characterized genes were preferred to ensure the markers associated with certain functions. Genes associated with above mentioned green characters were given priority.
- (6) As upland rice possesses abundant genetic resources in terms of many green characters (e.g. drought resistance, nitrogen use efficiency, direct seeding, etc.), which are adaptively differentiated from lowland rice, 2–3 upland-lowland differentiated SNP loci (difference in AF between typical upland and lowland rice >0.30) at the highly differentiated windows between typical upland and lowland rice ecotypes identified previously were designed (coded as *Geng* upland China and *Geng* lowland China) (Luo et al., 2022).

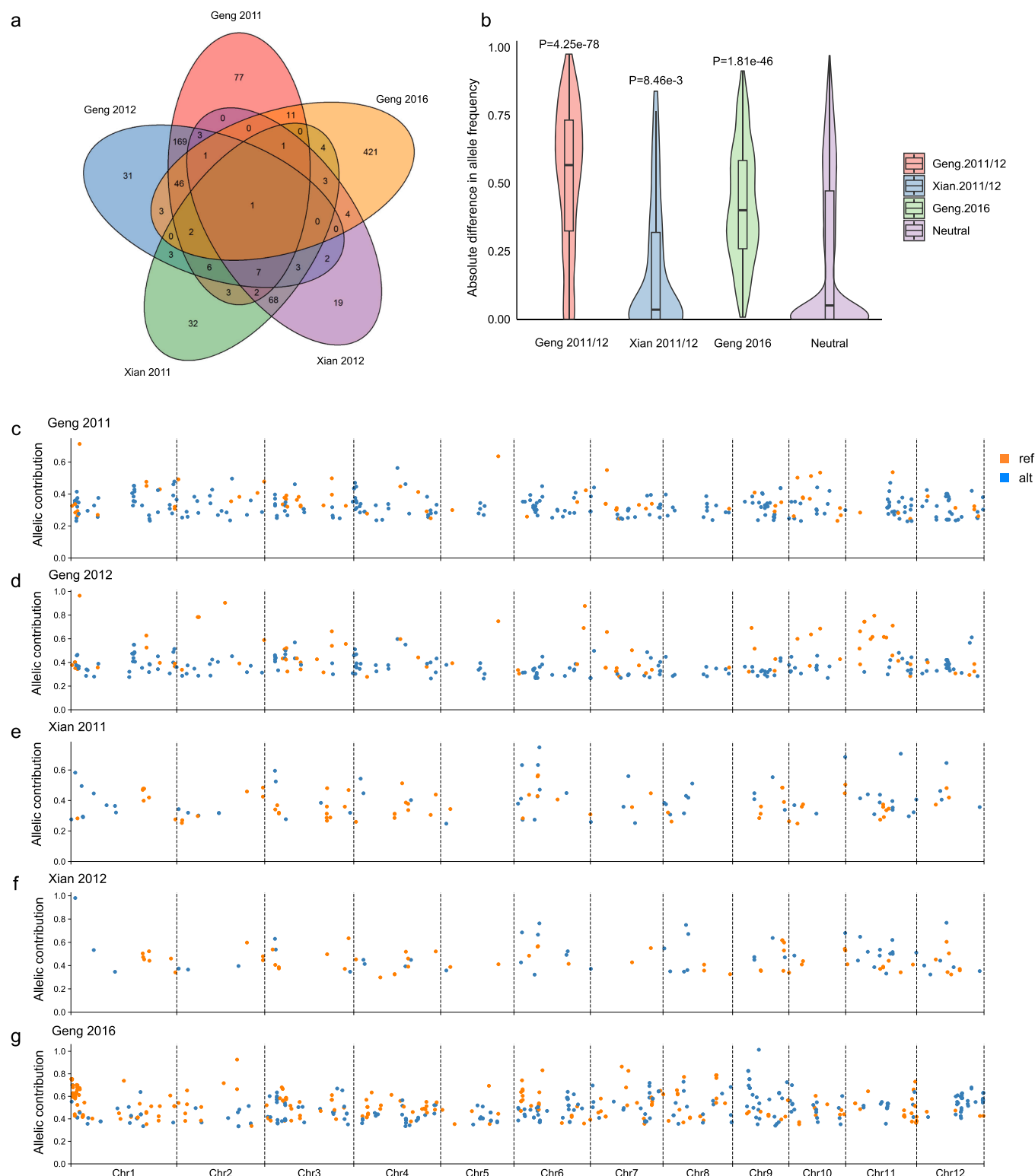


Fig. 5. Information of drought resistance contributing loci (DRCL) calculated from different datasets. **a.** Venn diagram of DRCL determined based on different datasets. **b.** Averaged absolute differences in allele frequencies between typical upland and lowland for DRCL based on different datasets. DRCL determined in 2011 and 2012 within the same subspecies are combined. $p < 0.05$ indicate the significance between DRCL and neutral loci based on independent t -test. **c-g.** Genomic distributions and their allelic contribution to drought resistances for the DRCL calculated from different datasets. The orange dot indicates the positive contribution from the reference allele (as the beneficial allele), while the blue dot indicates the positive contribution from the alternative allele (as the beneficial allele).

(7) Fifty-six SNP/InDel markers which are associated with agronomic important traits and widely used in rice breeding, were designed in this array (Table S2).

Following the above principles, 8822 SNP loci were initially screened out from the SNP source (>6000,000 SNP) generated from our experimental populations. After an estimation based on the optimized thermodynamic stability algorithm model for probe design and sequences

Table 4

Number of drought resistance contributing SNP loci in three 6K SNP arrays based on three field experiments.

Array	WDR6K		Rice6K-1		Rice6K-2	
	Xian	Geng	Xian	Geng	Xian	Geng
2011-DRC	134	329	70	121	137	144
2012-DRC	113	277	42	152	114	219
2016-RGW	NA	497	NA	237	NA	128

synthesis, 5895 SNP/InDel loci with 5994 capture probes (Table S3) were finally included in this breeding array. The SNP array, named WDR6K (ver. 1.0), was then developed by Huazhi Biotechnology Co., Ltd. (<http://en.higentec.com/>) using the liquid phase genotyping by pinpoint sequencing of captured targets (cGPS) technology.

To test the repeatability among technique replicates, two technique replicates were included in genotyping two rice genotypes (Nip2-1/-2 and SAGC7-1/-2). To test the repeatability among biological replicates, two independent individuals (as biological replicates) were included in genotyping Nipponbare (coded as Nip1 and Nip2) by WDR6K. To test the genotyping efficiency among rice genotypes. Forty rice genotypes were randomly selected and were genotyped by WDR6K.

Comparison among WDR6K, Rice6k-1, and Rice6k-2 in their SNPs and covered genes

Two rice SNP arrays previously developed for genetics and breeding applications in rice were used to compare with WDR6K. The C6AIR array (named Rice6K-1) contains 5274 SNP loci (Thomson et al., 2017), while RICE6K (named Rice6K-2) contains 5636 SNP/InDel loci (Yu et al., 2014). As the SNPs of Rice6K-1 and Rice6K-2 were based on different versions of the reference genome, we first converted them to the latest reference genome MSU7.0 (http://rice.uga.edu/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/pseudomolecules/version_7.0/), resulting in 4222 and 5617 SNP loci for Rice6K-1 and Rice6K-2, respectively. Types of the selected SNPs of WDR6K, Rice6K-1, and Rice6K-2 were analyzed and compared among the three arrays. Allele frequencies of the selected SNPs in the source population (where SNPs were selected from) and RICE3K population (Zhang et al., 2023) were calculated and compared among the three arrays. Additionally, as the differentiation between typical upland and lowland rice was associated with rice drought resistance (Xia et al., 2014; 2019; Luo et al., 2022), we also calculated the differences in allele frequencies between typical upland and lowland rice for genomic SNPs and SNP of WDR6K, Rice6K-1, and Rice6K-2. Finally, we applied the Principal Component Analysis (PCA) for the source population based on the genomic SNPs and WDR6K, respectively.

We further defined the SNP array covered gene when it contained at least one SNP from -2000 bp to 2000 bp of this gene. As a result, the three SNP arrays had distinguished pools of covered gene. KEGG and GO enrichments were then applied for the covered gene pools of WDR6K, Rice6K-1, and Rice6K-2 to test their differences in biological processes and/or pathways. Besides, involvements of the classified function-characterized genes (Table S4) in the array covered genes were counted and compared among the three arrays.

Commonly, if the expression of a gene is in responses to drought stress, it is more closely related to drought resistance. We further investigated gene expression changes of the array covered genes to a progressive field drought and treatments of 100 $\mu\text{mol/L}$ abscisic acid (ABA), 15 % PEG6000, 150 mmol NaCl , and 1 % H_2O_2 . The gene expression profile of the field transcriptomes of two rice genotypes (IRAT109 and Huhan1B) were extracted from a previous transcriptomic study (Xia et al., 2020). To collect the transcriptome data under above-mentioned treatments, two-week-old rice seedlings (Nipponbare) were used. The shoot samples in each treatment with three biological replicates were harvested at 2 h, 4 h, 12 h, and 24 hours respectively. We

extracted the total RNA using the PureLink® Plant RNA Reagent (Life Technologies). We used the qualified RNA samples for library construction following the specifications of the TruSeq® RNA Sample Preparation v2 Guide (Illumina) and conducted the RNA-sequencing with Illumina HiSeq 2500. We used SeqPrep to strip adaptors and/or merging paired reads with overlap into single reads (<https://github.com/jstjohn/SeqPrep>) and used Sickle to remove low-quality reads (<https://github.com/najoshi/sickle>). We then assembled the clean data using the software Cufflinks and mapped them to the reference genome MSU7.0 via Tophat with no more than two base mismatches allowed in the alignment. We determined the gene expression levels with the Fragment Per Kilobase of exon per Million fragments mapped (FPKM) method using the widely applied software Cuffdiff. Values of the absolute $\text{Log}_2^{\text{(Fold Change)}}$ were calculated for all genes (averaged FPKM >0.50) covered by the three arrays, respectively. Finally, the averaged absolute $\text{Log}_2^{\text{(Fold Change)}}$ for WDR6K, Rice6K-1, and Rice6K-2 were calculated and compared by independent *t*-test. The raw data were deposited in the NCBI Sequence Read Archive (PRJNA609211).

Genome-wide association study (GWAS) for drought resistance based on WDR6K

GWAS is an important application in genetic study. To test the applicability of WDR6K in GWAS to identifying potential QTLs of rice drought resistance, we combined the SNPs of the three arrays together and applied the GWAS. In addition, if more QTLs can be identified by GWAS using WDR6K, it mean high probability of its association with drought resistance.

The data of drought resistance quantified by the relative grain yield (RGW) or the drought resistance coefficient (DRC) was extracted from two previous independent studies. The first study contained 215 *Geng* landraces (10X in averaged sequencing depth) and their drought resistances quantified by RGW (calculated as grain yield in drought-stressed field/ grain yield in well-watered field) in field experiment in 2016 (Xia et al., 2019). The other study contained 270 rice genotypes (5X in averaged sequencing depth), whose drought resistances were quantified by DRC (Ma et al., 2016). DRC was also calculated as grain yield in drought-stressed field/ grain yield in well-watered field in field experiments in 2011 and 2012. The detail information about populations and experiments can be found in above mentioned studies (Ma et al., 2016; Xia et al., 2019).

The GWAS model was implemented using the GAPIT (Genome Association and Prediction Integrated Tool) R package, which employs the Efficient Mixed-Model Association (EMMA) method. The GAPIT package was developed by Zhang et al., (Zhang et al., 2010). The EMMA method as one of the statistical methods has been integrated into the GAPIT software package in R. The threshold was set at $p = 1/\text{total number of SNPs}$, which was widely used in plant GWAS studies (Ma et al., 2016; Bai et al., 2010). To apply the GWAS in this manuscript, we extracted the selected SNPs of the three arrays from the re-sequencing data.

Calculation of the allelic contribution to drought resistance for the selected SNP loci

Two above-mentioned experimental populations, whose drought resistance were previously evaluated, were used to calculate the allelic contribution to drought resistance for the selected SNP loci. The first population contained 215 *Geng* rice landraces whose drought resistance (referred as RGW) were evaluated in 2016 (Xia et al., 2019). The other population contained 270 rice genotypes combined with *Geng* and *Xian* subspecies, whose drought resistance (referred as DRC) were evaluated in 2011 and 2012.

The allelic contribution of a SNP to rice drought resistance were calculated as: (drought resistance of genotypes possessed reference allele - drought resistance of genotypes possessed alternative allele)/

averaged drought resistance of total genotypes. The positive value of the allelic contribution indicated the reference allele was beneficial, while the negative value indicated the alternative allele was beneficial. The drought resistance contributing loci (DRCL) were then defined when its absolute allelic contribution to drought resistance was $>20\%$ and the $p < 0.05$ between genotypes of reference and alternative alleles by independent t -test. To avoid the bias from genetic background, the DRCL was separately calculated within *Xian*, *Geng* (for DRC obtained in 2011 and 2012), and *Geng* lowland rice (for RGW obtained in 2016). As indicated by several previous report and hypothesized in this study, the highly differentiated loci between typical upland and lowland rice ecotypes should more likely be associated with drought resistance. To test this, we calculated the absolute AF difference between DTU and DTL for all the DRCL and neutral loci.

Results

General description of SNPs in WDR6K array

The selected 5985 SNP loci distributed evenly throughout the rice genome (Fig. 1a), with an averaged gap length of ~ 63.0 Kb between any two markers (Table 1). Most gaps between any two markers were <100 kb (Figure S1). This allows it applicative in the background selection. The biggest difference between WDR6K and Rice6K-1/2 was the great involvement of missense SNVs (including nonsynonymous, stoploss, and stop-gain SNVs), which accounted for $>90\%$ (Fig. 1b). In comparison, Rice6K-1 and Rice6K-2 both contained $<10\%$ missense SNVs (Fig. 1c, d). In addition, WDR6K, Rice6K-1, and Rice6K-2 shared very few common SNPs (Fig. 1e). As indicated by the AF of its SNPs among the source population and Rice3K population (Table 2), WDR6K had equivalent genetic diversity to Rice6K-1 and Rice6K-2, although contained a much higher proportion of missense SNVs. Moreover, WDR6K possessed great number (2358, accounting for 41.0% total SNPs) of DTU-DTL differentiated SNPs ($dAF \geq 0.30$), much more than those in Rice6K-1 and Rice6K-2 (Table 3). As a result, the separation of typical upland and lowland rice landraces by the PCA based on WDR6K (Fig. 2b) was better than those based on the genomic SNPs (Fig. 2a). In addition, WDR6K had good repeatability among technical (Table S5) and biological replicates (Table S6), as well as high efficiencies in rice genotyping (Table S7).

Genes covered by WDR6K and comparisons between those covered by Rice6K-1 and Rice6K-2

WDR6K covered 5001 genes, while rice6K-1 and rice6K-2 covered 2899 and 3516 genes, respectively. They were rarely overlapped (Fig. 3a). There were 2280 functionally characterized genes till December 31st, 2023 (Table S4). The coverage of known genes was much higher in WDR6K than those of rice6K-1 and rice 6K-2, particularly on these genes related to the green characters (e.g., direct seeding, drought resistance, salinity tolerance, heat tolerance, and nitrogen/phosphorus use efficiency) required by WDR breeding (Fig. 3b).

Based on the analysis of KEGG enrichment, we found the plant hormone signal transduction, MAPK signaling pathway, and plant-pathogen interaction were the top three highest enriched pathways by the gene pool of WDR6K. In contrast, the enriched pathways by gene pools of Rice6K-1 and Rice6K-2 were similar, which were closely related to energy metabolism, such as carbon metabolism, starch and sucrose metabolism, and glycolysis/ gluconeogenesis (Figure S2). Based on the GO enrichment, we can also find many GO terms specifically enriched by the gene pool of WDR6K, such as kinase activity, programmed cell death, defense response, etc. (Figure S3). In addition, genes covered by WDR6K represented higher fold changes in responses to the field drought and treatments of ABA, osmotic, salinity, and oxidative stresses, than those covered by Rice6K-1 and Rice6K-2 did (Fig. 3c, d). All these results indicated that the genes covered by WDR6K were more closely related to drought resistance and/or other green characters.

Contribution of WDR6K loci to rice drought resistance

First, we identified nine signals beyond the threshold ($1/n$) in total based on GWAS, five among which were of WDR6K (Fig. 4, Table S8). This result indicated that WDR6K possessed more drought resistance associated loci than other two rice 6K arrays based on GWAS and had the potential in genetic dissection of drought resistant QTLs.

Second, WDR6K possessed 922 DRCL according to their allelic contributions ($>20\%$ and $p < 0.05$) among diverse datasets (Fig. 5a, c-h), much more than those of other two rice 6K arrays (Table 4). We can also determine the beneficial allele of a locus by its allelic contribution. For example, the positive value of the allelic contribution indicated the reference allele (orange dots in Fig. 5c-g) was beneficial, while the negative value indicated the alternative allele (blue dots in Fig. 5c-g) was beneficial. As a result, we can select the beneficial (reference or alternative) allele in the foreground selection of drought resistance once it positively contributes to rice drought resistance. It is noteworthy that the AF difference between upland and lowland rice ecotypes of these DRCL within the *Geng* genetic background was much higher than that of neutral loci (Fig. 5b), indicating the upland-lowland differentiated locus contributed a lot to rice drought resistance than the neutral locus did. Eight-three upland-lowland differentiated DRCL in sixty-eight function genes, which can be used in the foreground selection of drought resistance, were listed in Table S9.

Discussion

The sustainable development of rice production requires resource-saving and environment-friendly varieties (Nawaz et al., 2022). WDR, an important type of “Green Super Rice” (Luo et al., 2019), has been well recognized in China (Yu et al., 2021). It performs well in the poor irrigated low-to-medium-yield fields with direct-seeding and water-saving cultivation (Xia et al., 2022). Its annual planting area has exceeded 260,000 hectares since 2023. The integration of green characters (e.g., direct seeding, drought resistance, water use efficiency, and nitrogen/phosphorus use efficiency, etc.) from upland rice into the elite paddy rice ensures the well adaptation of WDR to the dry cropping system without irrigation (Xia et al., 2022; Bi et al., 2021). In other words, the molecular design breeding of WDR requires molecular markers associated with the above-mentioned green characters, particularly the drought resistance to face the climate change. Therefore, a SNP array which can assist the foreground selection of drought resistance and other green traits, as well as the background selection is required.

The design of WDR6K (ver. 1.0) is mainly served for the foreground selection of drought resistance and some other green traits from upland rice. The first notable feature of WDR6K is the great involvement of missense SNP loci ($>90\%$). Other rice SNP arrays (Singh et al., 2015; Yu et al., 2014; Thomson et al., 2017; Zhao et al., 2011; Chen et al., 2014; McCouch et al., 2016), including the two rice 6K SNP arrays (Yu et al., 2014; Thomson et al., 2017) used in comparisons with WDR6K, always have more (68% to 88%) assumed neutral loci (intergenic, intronic, and synonymous SNPs) to ensure the polymorphism. This makes the markers of WDR6K are more likely to be associated with certain phenotypes or functions. It is also noteworthy that the genetic diversity is not apparently affected by the selection of missense SNPs in WDR6K, making it well applied in the genetic study and background selection.

Second, the genes covered by WDR6K are different from other rice 6K SNPs. For example, genes covered by WDR6K are associated with special GO terms (e.g., kinase activity, programmed cell death, defense response, etc.) and KEGG pathways (e.g., plant hormone signal transduction and MAPK signaling pathway). Moreover, genes covered by WDR6K have greater fold changes in responses to drought, osmotic, oxidative, salinity stresses, and the plant phytohormone ABA. All these results indicate that markers in WDR6K have higher probabilities to be associated with drought resistance. In addition, based on the progress of

rice functional genomics, WDR6K covers more function-characterized genes, particular these associated with these green traits required by WDR breeding. Therefore, the design of WDR6K allows it to be well applied in the foreground selection in drought resistance and other related green traits.

The selection of upland-lowland differentiated loci is another important feature of WDR6K which let it different from other rice SNP arrays. Many upland rice dominant/specific alleles of many genes associated with these green characters have been proven beneficial in breeding (Li et al., 2023; Wang et al., 2024; Han et al., 2024; Li et al., 2015; Yang et al., 2021). Interestingly, the upland-lowland differentiated loci designed in WDR6K provide a lot of drought resistance contributing loci for the foreground selection of drought resistance. For example, qRT9 is a well-recognized locus that has been highly differentiated between upland and lowland rice and is clearly associated with rice drought resistance by regulating root architecture (Li et al., 2015; Wang et al. 2024). In this study, its allelic contributions were negative ($-0.270 \sim -0.419$ in Table S9), indicating the upland-dominant alternative allele was the beneficial allele in the foreground selection. This result is consistent to the previous studies (Li et al., 2015; Wang et al. 2024), which proves the validity of our strategies in the design of WDR6K by introducing the upland-lowland differentiated loci.

Finally, the application of cGPS technology can not only reduce the cost of genotyping by the SNP array, but also provide further flexibilities in update SNP loci to develop advanced version. The cost of one test by WDR6K is <50 Chinese Yuan (~ 7.0 US dollars) with nearly 6000 SNP loci which is sufficient for both background selection and foreground selection. In addition, we can add additional markers into WDR6K once we have learned more about genetic bases of drought resistance and associated markers to update the version of WDR6K. Actually, the true effects of these DRCLs in breeding still requires further validations. It is because the drought resistance is a complex trait and the allelic contribution of a locus varies a lot among populations (genetic backgrounds) and environments (experiments) as indicated by this study. For example, a locus which contributes a lot in the *Geng* subspecies may contribute a little in the *Xian* subspecies. Similar observations are also represented among different experiments. As a result, efforts should be made to generate breeding modules of large effects with greater data.

In summary, WDR6K (ver. 1.0) is designed for molecular designed breeding of WDR with the special SNP selection strategy including great amounts of missense SNPs and upland-lowland differentiated loci. This makes WDR6K markers are closely associated with drought resistance and other green traits. We can use WDR6K to diagnose the presence and/or absence of beneficial alleles in recurrent and donor lines. We can then apply foreground selections in breeding WDR by pyramiding beneficial alleles that contributes to the drought resistance. Therefore, WDR6K is low-cost, efficient, and developing and has great prospective in molecular designed breeding of WDR.

CRedit authorship contribution statement

Ziming Li: Writing – original draft, Validation, Investigation, Formal analysis, Data curation. **Lei Wang:** Software, Formal analysis, Data curation, Conceptualization. **Yi Liu:** Project administration, Methodology, Funding acquisition, Conceptualization. **Xiaosong Ma:** Data curation, Conceptualization. **Anning Zhang:** Project administration, Methodology, Conceptualization. **Zhi Luo:** Software, Methodology, Formal analysis. **Ming Yan:** Visualization, Methodology. **Liguo Zhou:** Resources, Project administration, Methodology. **Liang Chen:** Supervision, Project administration. **Lijun Luo:** Writing – review & editing, Supervision, Funding acquisition. **Hui Xia:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Hui Xia reports financial support was provided by Shanghai and Natural Science Foundation of china. Anning Zhang reports financial support was provided by China Agriculture Research System-Rice. Lijun Luo reports financial support was provided by Shanghai Agriculture Applied Technology Development Program. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at [doi:10.1016/j.stress.2025.100800](https://doi.org/10.1016/j.stress.2025.100800).

Data availability

Data will be made available on request.

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