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# A major QTL controlling deep rooting on rice chromosome 4

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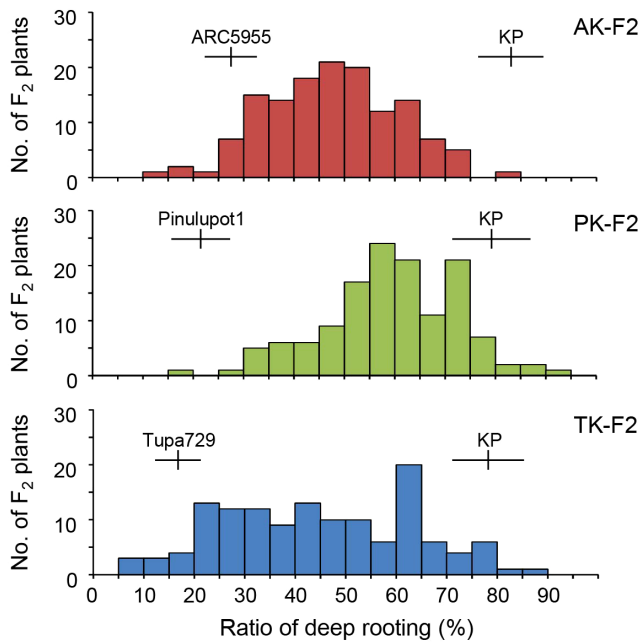
Drought is the most serious abiotic stress that hinders rice production under rainfed conditions. Breeding for deep rooting is a promising strategy to improve the root system architecture in shallow-rooting rice cultivars to avoid drought stress. We analysed the quantitative trait loci (QTLs) for the ratio of deep rooting (RDR) in three  $F_2$  mapping populations derived from crosses between each of three shallow-rooting varieties ('ARC5955', 'Pinulupot1', and 'Tupa729') and a deep-rooting variety, 'Kinandang Patong'. In total, we detected five RDR QTLs on chromosomes 2, 4, and 6. In all three populations, QTLs on chromosome 4 were found to be located at similar positions; they explained from 32.0% to 56.6% of the total RDR phenotypic variance. This suggests that one or more key genetic factors controlling the root growth angle in rice is located in this region of chromosome 4.

Global climate change in recent years has caused serious drought damage to crop production, resulting in higher food prices, lower farm income, and more food insecurity. Therefore, strategies for the genetic enhancement of drought resistance in crop plants are essential for a stable and adequate food supply. Rice (*Oryza sativa* L.), a staple food for nearly half of the world's population, is susceptible to drought stress because of its shallow rooting compared with other cereal crops and therefore a limited capacity to extract water from deep soil layers<sup>1,2</sup>. Nevertheless, rice is frequently exposed to drought in 20% of its total growing area in Asia<sup>3</sup>. Many studies have suggested that a deep root system helps plants to avoid drought stress by extracting water from deep soil layers<sup>4-6</sup>. Typical upland rice has deeper rooting than lowland rice<sup>7</sup>. The deep root system in upland rice may contribute to its drought avoidance through enhanced water uptake<sup>8</sup>. Therefore, to ensure drought avoidance in shallow-rooting rice, introduction of genes responsible for deep rooting into such rice cultivars is considered as one of the most promising breeding strategies<sup>6</sup>.

Deep rooting is a complex trait because the vertical root distribution is determined by a combination of the root growth angle and maximum root length in seminal and nodal roots of cereal crops<sup>9,10</sup>. Thus, the development of deep roots requires multiple genes that confer near-vertical root growth axis and increase root length along this axis. According to Courtois *et al.*<sup>11</sup>, a total of 103 quantitative trait loci (QTLs) for root length have been reported in rice. Recently, Obara *et al.*<sup>12</sup> fine-mapped *qRL6.1*, a QTL located on chromosome 6 and associated with root length in hydroponically grown rice seedlings. However, there have been few reports of mapped QTLs associated with root growth angle in rice. *DRO1*, a QTL for deep rooting on chromosome 9<sup>13</sup>, and *qSOR1*, a QTL for soil-surface rooting on chromosome 7<sup>14</sup> have been mapped. Both QTLs contribute considerably to the root growth angle. Cloning and characterization of *DRO1* showed that it functions downstream of the auxin signalling pathway and controls the gravitropic curvature in rice roots<sup>15</sup>. Under upland conditions with drought stress, a near-isogenic line (NIL) with a functional allele of *DRO1* introduced from the deep-rooting cultivar 'Kinandang Patong' has deeper roots and a significantly higher grain yield than the shallow-rooting parent variety 'IR64', which has a non-functional allele of *DRO1*. Thus, *DRO1* is the first gene associated with root system architecture (RSA) that has been shown to improve the ability to avoid drought.

Sequence analysis of the *DRO1* transcribed regions in 64 rice cultivars revealed six haplotypes<sup>15</sup>. Interestingly, some shallow-rooting cultivars grouped together with the cultivars carrying a functional allele of *DRO1* such as 'Kinandang Patong', suggesting the existence of undetected QTL(s) associated with root growth angle distinct from *DRO1*. Availability of multiple genes that affect the root growth angle would be useful for breeding strategies using marker-assisted selection (MAS) to develop rice cultivars with high adaptability to drought.

To identify new QTLs for root growth angle in rice, we selected three accessions (other than 'IR64') with the shallowest rooting from different *DRO1* haplotype groups based on our previous study<sup>15</sup>. 'Tupa729' belongs to Group I together with 'Kinandang Patong'. 'ARC5955' and 'Pinulupot1' belong to Groups II and IV, respectively. We conducted QTL analyses for the root growth angle in three  $F_2$  mapping populations derived from a cross between each shallow-rooting variety and 'Kinandang Patong' as a donor line with deep roots.



**Figure 1** | Frequency distribution of the ratio of deep rooting in the three  $F_2$  populations: AK-F2, ‘ARC5955’  $\times$  ‘Kinandang Patong’ (KP); PK-F2, ‘Pinulupot1’  $\times$  ‘Kinandang Patong’; TK-F2, ‘Tupa729’  $\times$  ‘Kinandang Patong’. Vertical and horizontal lines above the bars indicate the mean and SD of each parental line.

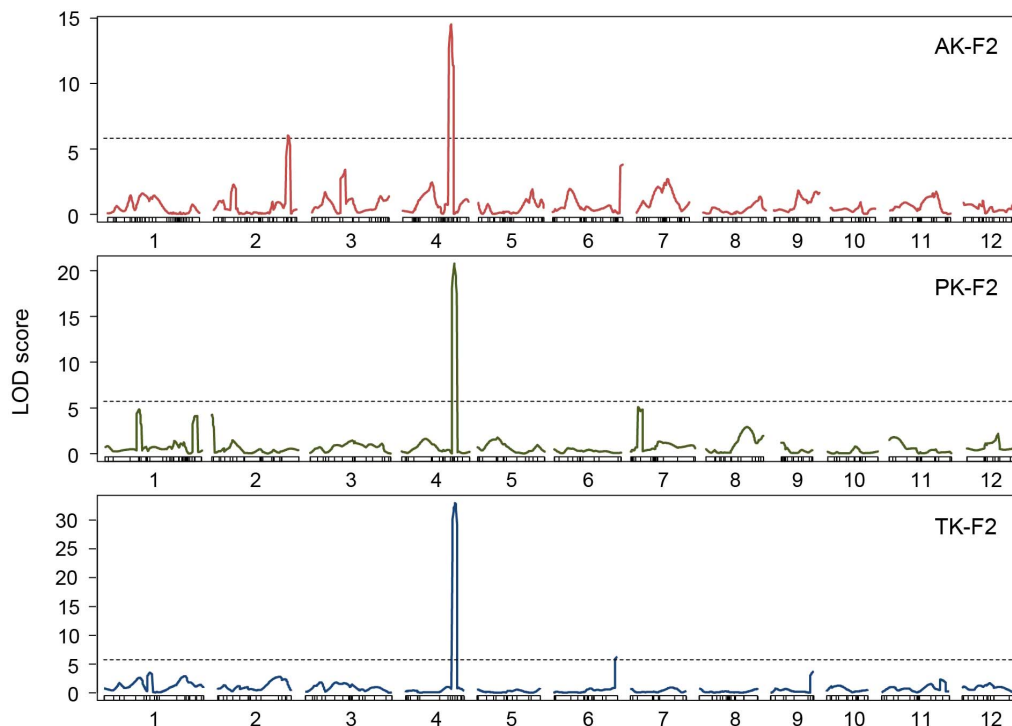
## Results

**Phenotypic variations of the ratio of deep rooting (RDR) in the three mapping populations.** Comparison of the RDR among the parental lines confirmed shallow rooting in ‘ARC5955’, ‘Pinulupot1’, and ‘Tupa729’ (ranging from 16.5 to 27.4%), whereas ‘Kinandang

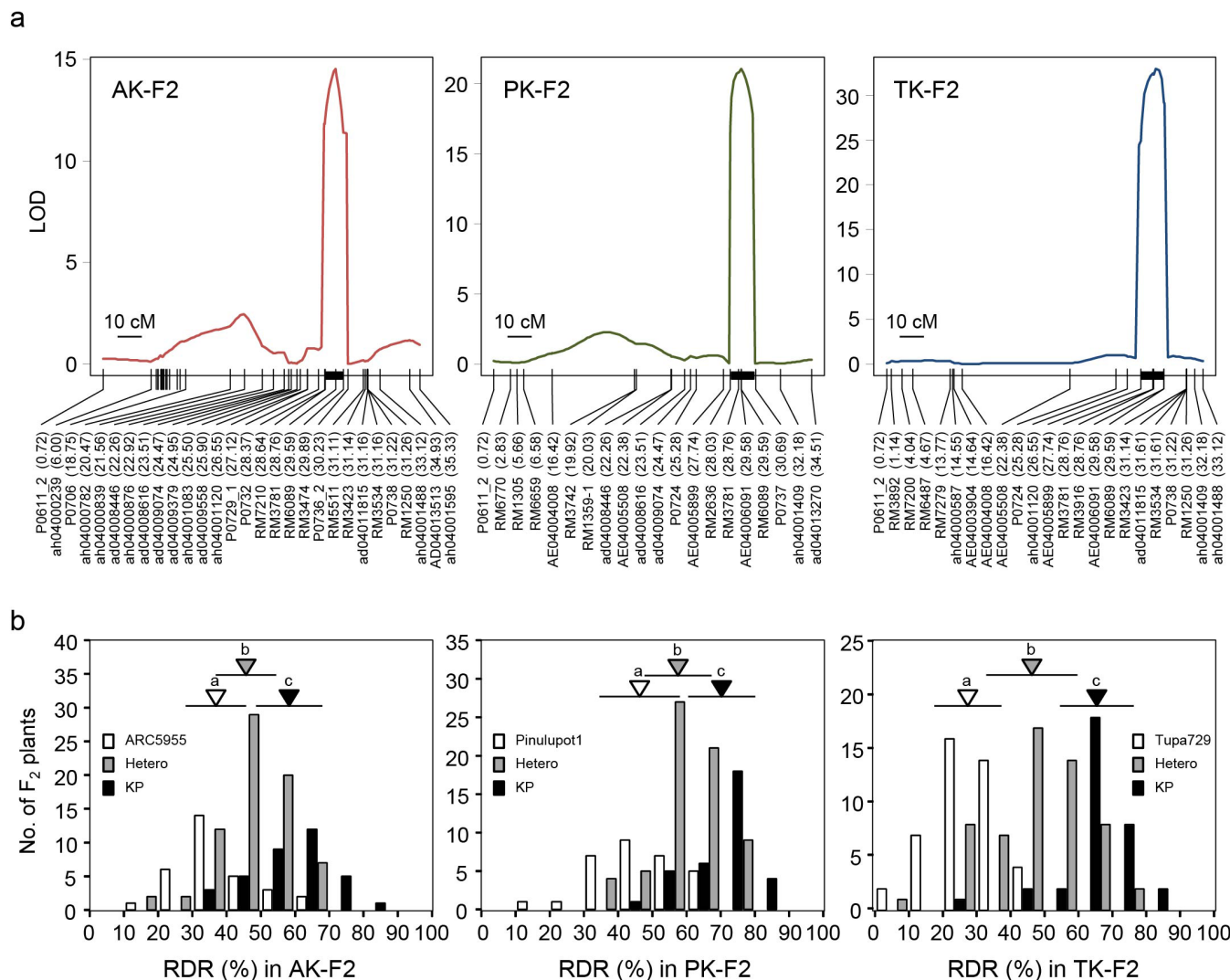
Patong’ showed deep rooting (approximately 80%) (Fig. 1). The RDR values in the three  $F_2$  mapping populations from the crosses between ‘Kinandang Patong’ and ‘ARC5955’ (AK-F2), ‘Pinulupot1’ (PK-F2), and ‘Tupa729’ (TK-F2) were distributed between the values of the respective parental lines without considerable transgressive segregation (Fig. 1). The broad-sense heritability ( $h^2_B$ ) of RDR was 79.9% in AK-F2, 73.6% in PK-F2, and 88.9% in TK-F2.

**Detection of QTLs for deeper rooting in AK-F2.** Among 768 single-nucleotide polymorphism (SNP) markers, 346 (45.1%) were polymorphic between ‘ARC5955’ and ‘Kinandang Patong’. To resolve the linkage gaps and to saturate the regions around the LOD support intervals for detected QTLs with DNA markers, we added 16 polymorphic simple sequence repeat (SSR) markers in these regions. The AK-F2 linkage map, composed of 362 markers, covered almost the entire rice genome (Supplementary Fig. 1). The total map length was 1533.5 cM, and the average distance between markers was 4.38 cM. Two QTLs for RDR on chromosomes 2 and 4 were detected based on an LOD threshold of 5.96 (Figs. 2 and 3a; Supplementary Fig. 1). The QTL on chromosome 4 had a large contribution to the phenotypic variance, explaining 32.0% of the total (Table 1). The mean RDRs of the lines homozygous for the ‘Kinandang Patong’ allele at the marker (RM6089) closest to the detected QTL were significantly higher than those of the lines homozygous for the ‘ARC5955’ allele (Fig. 3b). The ‘Kinandang Patong’ allele at this QTL increased the RDR. The minor QTL on chromosome 2 explained 9.5% of total phenotypic variance (Fig. 2; Table 1; Supplementary Fig. 1). A two-dimensional scan revealed no significant epistatic interaction in the whole genome in this population (Supplementary Fig. 2).

**Detection of QTLs for deeper rooting in PK-F2.** Among 768 SNP markers, 184 (24.0%) were polymorphic between ‘Tupa729’ and ‘Kinandang Patong’. We also mapped 53 polymorphic SSR markers. The PK-F2 linkage map, composed of 237 markers, covered almost the entire rice genome (Supplementary Fig. 3). The total map length



**Figure 2** | LOD score curves for the QTLs for the ratio of deep rooting in the three  $F_2$  populations (see Fig. 1 for designations). Rectangles represent linkage maps with DNA marker positions shown as vertical lines. Chromosome numbers are indicated under each linkage map (short arms are on the left). Dotted lines indicate LOD thresholds (AK-F2, 5.96; PK-F2, 5.72; TK-F2, 5.46).



**Figure 3 | Chromosomal positions and allelic effects of the QTLs for the ratio of deep rooting (RDR) on chromosome 4 detected in the three F<sub>2</sub> populations (see Fig. 1 for designations).** (a) The peaks of the LOD curves indicate the putative positions of RDR QTLs. Vertical lines in each linkage map indicate the genetic positions (cM) of DNA markers. Black bars on the linkage maps indicate 1.8-LOD support intervals calculated by using the lodint function in R/qtl<sup>36</sup>. DNA markers are shown under the linkage maps; the numbers in parentheses indicate their physical map positions (Mb) on chromosome 4 of ‘Nipponbare’. (b) Frequency distribution of RDR in three F<sub>2</sub> populations showing three genotype classes of the DNA markers closest to the RDR QTLs (RM6089 in AK-F2 and PK-F2; RM3916 in TK-F2). The inverted triangles indicate the mean, and the horizontal bars indicate SD of each allele. The same shading is used for triangles and corresponding bars. The means labelled with different letters differ significantly among the three alleles ( $P < 0.05$ , Tukey’s multiple comparison test).

Table 1   Putative QTLs for ratio of deep rooting detected in three F <sub>2</sub> populations									
Population	Chr.	Nearest marker	Mb	cM	LOD	AE	DE	R <sup>2</sup>	
AK-F2	2	AE02004954	32.30	0.0	6.00	5.8	-0.5	9.5	
	4	RM6089	29.59	0.0	14.50	10.6	-1.4	32.0	(39.8)
PK-F2	4	RM6089	29.59	0.0	20.80	11.3	-0.3	36.2	
TK-F2	4	RM3916	28.76	0.8	32.98	18.9	-0.4	56.6	
	6	AD06014592	31.20	0.0	6.27	6.7	0.2	6.3	(68.3)

Mb: Physical map position of each marker based on the RAP database (<http://rapdb.dna.affrc.go.jp/>). cM: Genetic distance from the QTL LOD peak to the nearest marker. AE: Additive effect of the allele from ‘Kinandang Patong’ compared with that from the paternal line. DE: Dominance effect of the allele from ‘Kinandang Patong’ compared with that from the paternal line. R<sup>2</sup>: Percentage of the phenotypic variance explained by each QTL. Numbers in parentheses indicate percentage of the phenotypic variance explained by multiple QTL.



was 1560.1 cM, and the average distance between markers was 6.93 cM. Only one QTL (on chromosome 4) was found based on a LOD threshold of 5.72 (Figs. 2 and 3a; Supplementary Fig. 3). This QTL had a large contribution to the phenotypic variance, explaining 36.2% of the total (Table 1). The mean RDRs of lines homozygous for the ‘Kinandang Patong’ allele at the marker (RM6089) closest to the detected QTL were significantly higher than those of the lines homozygous for the ‘Pinulupot1’ allele (Fig. 3b). The Kinandang Patong allele at this QTL increased the RDR. A two-dimensional scan revealed no significant epistatic interaction in the whole genome in this population (Supplementary Fig. 4).

**Detection of QTLs for deeper rooting in TK-F2.** Among 768 SNP markers, 160 (20.8%) were polymorphic between ‘Pinulupot1’ and ‘Kinandang Patong’. We also mapped 64 polymorphic SSR markers. The TK-F2 linkage map, composed of 224 markers, covered almost the entire rice genome (Supplementary Fig. 5). The total map length was 1434.1 cM, and the average distance between markers was 6.76 cM. Two QTLs were detected on chromosomes 4 and 6 based on an LOD threshold of 5.46 (Figs. 2 and 3a; Supplementary Fig. 5). The QTL on chromosome 4 had a very large contribution to the phenotypic variance, explaining 56.6% of the total (Table 1). The mean RDRs of lines homozygous for the ‘Kinandang Patong’ allele at the marker (RM3916) closest to the detected QTL were significantly higher than those of lines homozygous for the ‘Tupa729’ allele (Fig. 3b). The ‘Kinandang Patong’ allele at this QTL increased the RDR. A QTL detected on chromosome 6 showed a small genetic effect, explaining only 6.3% of total phenotypic variance (Fig. 2; Table 1; Supplementary Fig. 5). A two-dimensional scan revealed no significant epistatic interaction in the whole genome in this population (Supplementary Fig. 6).

## Discussion

To identify new QTLs for root growth angle in rice, we selected three accessions with shallow rooting from different varietal groups of cultivated rice, and produced F<sub>2</sub> mapping populations derived from their crosses with ‘Kinandang Patong’. We detected two QTLs for RDR in AK-F2, two in TK-F2, and one in PK-F2. These are novel QTLs for root growth angle because their chromosomal positions differed from those reported in our previous studies<sup>13,14</sup>. The three QTLs on chromosome 4, detected in the three populations, showed very high LOD values and a large contribution to the total phenotypic variance (Figs. 2 and 3; Table 1). The physical positions of the markers most closely linked to the LOD peaks of these QTLs indicated that they may correspond to the same locus. Therefore, we assume that one or more key genes responsible for root growth angle may be located in this chromosome region, and we designate this QTL as *DEEPER ROOTING 2* (*DRO2*). The ‘Kinandang Patong’ allele of *DRO2* increased the RDR in all three populations compared with the alleles from the three shallow-rooting parents. The ‘Kinandang Patong’ allele of *DRO1*, detected in the ‘IR64’ × ‘Kinandang Patong’ recombinant inbred lines (IK-RILs), also resulted in deeper roots than the ‘IR64’ allele<sup>13</sup>. These results suggest that deep rooting of ‘Kinandang Patong’ is largely controlled by *DRO1* and *DRO2*, although it is unknown whether these QTLs interact epistatically. To clarify the genetic interaction between them, further analysis using NILs for *DRO1* and *DRO2* will be needed.

We compared the relationship between the positions of *DRO2* and other root QTLs by using the QTL Annotation Rice Online Database (Q-TARO, <http://qtaro.abr.affrc.go.jp/>)<sup>16</sup> and the data summarized by Courtois *et al.*<sup>11</sup>. We found only one QTL for deep root ratio (percentage of the volume of deep roots [below 30 cm] relative to the total root volume), a parameter related to RDR, in the LOD support interval for *DRO2* (Fig. 3a; 27.74–30.69 Mb based on the ‘Nipponbare’ genome sequence in the RAP database; <http://rapdb.dna.affrc.go.jp/>). This QTL was located between the SSR markers

RM470 (28.28 Mb) and RM317 (29.25 Mb) in RILs derived from a cross between a lowland rice cultivar ‘Zhenshan97’ (*indica*) and an upland cultivar ‘IRAT109’ (tropical *japonica*)<sup>17</sup>, but comparison of the marker positions alone is not sufficient to determine whether *DRO2* is related to this QTL. We also found that a QTL for root volume (*qFSR4*)<sup>18</sup> had been fine-mapped near the candidate region of *DRO2*. However, the candidate region of *qFSR4* is located in the long arm at >31 Mb<sup>18</sup>, and thus is outside of the LOD support interval for *DRO2*; this excludes *qFSR4* as a candidate for *DRO2*. According to the report of Courtois *et al.*<sup>11</sup>, many QTLs for other root traits (but not root growth angle) have also been detected in the putative region of *DRO2*. Further study using a NIL for *DRO2* should be conducted to clarify whether *DRO2* has pleiotropic effects on other root morphology traits. For this purpose, we are currently developing a NIL for *DRO2* in each of the shallow-rooting varieties.

We found no genes homologous to *DRO1* in the *DRO2* LOD support interval. This suggests that *DRO1* and *DRO2* are not structurally related. Many auxin-related genes such as *CROWN ROOTLESS1* (*CRL1*)/*ADVENTITIOUS ROOTLESS1*<sup>19,20</sup>, *OsPID1*<sup>21</sup>, *CRL4/OsGNOM1*<sup>22,23</sup>, and *CRL5*<sup>24</sup> are associated with root gravitropism, which determines the root growth angle. Therefore, we checked the *DRO2* LOD support interval for the presence of auxin-related genes, but did not find any candidate genes in this region. We also compared the positions of *DRO2* and known root genes characterized in mutants by using the Overview of functionally characterized Genes in Rice Online database (OGRO, <http://qtaro.abr.affrc.go.jp/ogro/>)<sup>25</sup>. We found three root-related genes, *DWARF17*<sup>26</sup>, *Oryza sativa indeterminate domain 10*<sup>27</sup>, and *GLR3.1*<sup>28</sup>, in the *DRO2* LOD support interval, but none of them has been reported to affect the root growth angle. Therefore, *DRO2* might be distinct from these known genes.

Sequences of the *DRO1* transcribed regions of 64 rice cultivars have been grouped into six haplotypes<sup>15</sup>. The three shallow-rooting varieties used in this study belong to different haplotype groups: Group I (‘Tupa729’ together with deep-rooting ‘Kinandang Patong’), Group II (‘ARC5955’) and Group IV (‘Pinulupot1’). The variety of Group I haplotype showed functional allele of *DRO1* in the previous study<sup>15</sup>. Therefore, we expected to detect QTLs other than *DRO1* in TK-F2. Indeed, we detected two new QTLs (but not *DRO1*) accounting for 68.3% of the total RDR phenotypic variance in TK-F2 (Table 1). This suggests that the differences in RDR between ‘Tupa729’ and ‘Kinandang Patong’ are mostly explained by these two new QTLs. On the other hand, we expected that *DRO1* might be detected in AK-F2 and PK-F2 because ‘ARC5955’ and ‘Pinulupot1’ belong to different *DRO1* haplotype groups than ‘Kinandang Patong’. However, *DRO1* was not found in these mapping populations. In primary mapping populations such as F<sub>2</sub>, segregation of major QTLs often masks the presence of minor QTLs and hampers their statistical detection<sup>29</sup>. We assume that *DRO1* could not be detected in the present study because of a small difference in the genetic effects of the ‘Kinandang Patong’ *DRO1* allele and those of ‘ARC5955’ or ‘Pinulupot1’. This result also suggests that Group II and IV have functional allele of *DRO1*. The detected QTLs in AK-F2 and PK-F2 contributed to only 39.8% and 36.2% of the total RDR phenotypic variance, respectively. The *h*<sup>2</sup><sub>B</sub> of RDR in IK-RILs was 77.7%<sup>13</sup>. In this study, AK-F2 and PK-F2 showed similar *h*<sup>2</sup><sub>B</sub>; no significant epistatic interaction could be detected in either population. Therefore, we believe that other QTLs in addition to *DRO2* were associated with the RDR in these two mapping populations but were not detected in this analysis. For instance, we observed two additional LOD peaks in AK-F2 at lower LOD threshold (LOD = 3.40 between ah03001245 and ah03001697 on chromosome 3; LOD = 3.78 at AD06014592 on chromosome 6) (Fig. 2; Supplementary Fig. 1). In PK-F2, we found four additional LOD peaks at lower LOD threshold (LOD = 4.80 at P0634\_1 and LOD = 4.09 at ah01003209 on chromosome 1; LOD = 4.24 between





AE02000044 and P0221\_3 on chromosome 2; LOD = 5.08 between ad07001335 and ad07001571 on chromosome 7) (Fig. 2; Supplementary Fig. 3). Chromosome segment substitution lines are very powerful tools for detection of minor QTLs because they enable separation of minor QTLs from major QTLs located in other chromosome regions<sup>29</sup>. Such advanced materials will be useful to elucidate whether favorable alleles of *DRO1* and other QTLs are present in AK-F2 and PK-F2.

We conclude that *DRO2* is a promising QTL that can be used to enhance drought avoidance of shallow-rooting rice cultivars by controlling the RSA, because the ‘Kinandang Patong’ allele of *DRO2* improves the RDR of cultivars in three different genetic backgrounds: *aus*, *indica*, and *japonica*. *DRO2* cloning will accelerate elucidation of its molecular function, and *DRO2* haplotype analysis using many rice accessions will enable breeding for a better RSA through MAS. Map-based cloning of *DRO2* is currently underway in our laboratory.

## Methods

**Plant materials.** For QTL analysis, we developed three F<sub>2</sub> populations from crosses between ‘Kinandang Patong’ and (i) ‘ARC5955’ (138 plants; AK-F2), (ii) ‘Pinulupot1’ (134 plants; PK-F2), and (iii) ‘Tupa729’ (133 plants; TK-F2). ‘ARC5955’ (NIAS Acc. No. WRC35) is a landrace (*aus*) of Indian origin. ‘Pinulupot1’ (NIAS Acc. No. WRC24) is a landrace (*indica*) of Philippine origin. ‘Tupa729’ (NIAS Acc. No. WRC55) is a landrace (tropical *japonica*) of Bangladeshi origin. ‘Kinandang Patong’ (International Rice Research Institute Acc. No. IRGC23364) is a landrace (tropical *japonica*) of Philippine origin.

**RDR measurement.** RDR was evaluated quantitatively (as an index for root growth angle) based on a basket method that uses open stainless-steel mesh baskets (top diameter of 7.5 cm and depth of 5.0 cm; PROUD, Ushiku, Japan), as described previously<sup>13</sup>. We defined the RDR as the number of roots that penetrated the lower part of the mesh (i.e.,  $\geq 50^\circ$  from the horizontal, centred on the stem of the rice plant) divided by the total number of roots that penetrated the whole mesh. Larger RDR values correspond to greater root growth angles.

**DNA marker analysis.** We determined the genotypes of F<sub>2</sub> populations by using SNP markers selected from genome-wide SNP marker data<sup>30,31</sup>, and SSR markers selected on the basis of the data from the International Rice Genome Sequencing Project<sup>32</sup>. Total DNA was extracted from leaves by using the CTAB method<sup>33</sup>. The SNPs were detected from a set of 768 custom SNP panels by using a GoldenGate Genotyping Universal-32 768-plex Assay Kit and the BeadStation 500 G system (both from Illumina, San Diego, CA, USA) according to the manufacturer’s instructions. PCR amplification was performed in 5- $\mu$ l reaction mixtures containing 0.5  $\mu$ l (20 ng) DNA, 1.0  $\mu$ l 5 $\times$  PCR buffer, 0.1  $\mu$ l 10 mM dNTPs, 0.025  $\mu$ l (5 units) KAPA2G Fast DNA Polymerase (Kapa Biosystems, Boston, MA, USA), 0.125  $\mu$ l of a mixture of forward and reverse primers (20 pM each), and 3.25  $\mu$ l H<sub>2</sub>O. The PCR programme consisted of an initial denaturation for 1 min at 95°C; followed by 35 cycles of 10 s at 95°C, 10 s at 55°C, and 1 s at 72°C; followed by a final extension for 30 s at 72°C. PCR products were separated by electrophoresis in 3% agarose gels (Type I agarose; Sigma-Aldrich, St. Louis, MO, USA) at 150 V for 120 min.

**Statistical and QTL analyses.**  $h^2_B$  of RDR was estimated from the variances of the parental plants and the variance among the F<sub>2</sub> lines, and calculated as

$$h^2_B[F_2] = (Vp[F_2] - \{Vp[P_1] + Vp[P_2]\})/2/Vp[F_2],$$

where  $Vp[F_2]$  is the phenotypic variance among F<sub>2</sub> lines, and  $Vp[P_1]$  and  $Vp[P_2]$  are the phenotypic variances of the parental plants.

Linkage maps were constructed from the genotype data in MAPMAKER/EXP 3.0 software<sup>34</sup>. Genetic distances were estimated by using the software’s Kosambi map function<sup>35</sup>. QTL analysis was performed by using the composite interval mapping function of the R/qtl software<sup>36</sup>. The composite interval mapping threshold was based on the results of 1000 permutations at a 5% significance level<sup>37</sup>. The confidence intervals around each significant QTL peak were determined by 1.8-LOD support intervals<sup>36</sup>. The additive and dominant effects and the percentage of phenotypic variance explained by each QTL ( $R^2$ ) at the maximum LOD score were estimated by using the sim.gen, makeqtl, and fitqtl functions in R/qtl<sup>36</sup>. Two-dimensional scans with a two-QTL model were conducted in R/qtl with the thresholds based on the results of 1000 permutations at a 5% significance level<sup>36</sup>.

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## Author contributions

Y.U. designed the experiments and wrote the manuscript. Y.U. and N.K. evaluated RDR. N.K. and S.K. performed SSR analysis. T.M. and S.F. performed SNP analysis. Y.U. and E.Y. performed QTL analysis.

## Additional information

**Supplementary information** accompanies this paper at <http://www.nature.com/scientificreports>

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