

Article

The Adenine at the 4th Exon of the *DRO1* Gene Provides Drought-Tolerance Capacity to Hybrid Rice Deyou4727 and Its Maintainer Line Dexiang074B

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Abstract: Extreme weather and global warming are drastic agricultural issues causing water scarcity. Under these conditions, it is vital to select drought-tolerant hybrid super rice breeds to enhance the rice yield in dry areas. In the current study, a dry-shed experiment was carried out in 2019–2020 to analyze the root growth, gene expression, and enzyme activity of hybrid rice Deyou4727, its parent (Dexiang074B * Chenghui727), taking IR64 as a universal control. Results showed that Deyou4727 and its maintainer line Dexiang074B produced more roots (30–50 cm soil) and higher enzymatic activity than IR64 during the drought stress period, indicating a good drought-tolerant capacity for Deyou4727. The distribution and morphology of the root system in Deyou4727 were inherited by the maintainer line Dexiang074B, which was confirmed by the high upregulation of *DRO1* and *OsPIN1b* gene expression and the sharing of the same ORF region in both the hybrid rice varieties. However, the *DRO1* gene ORF study showed that, in the case of the control IR64, 'A' was missing at the 4th exon of the *DRO1* gene, which may explain the shallow and weak root architecture system of IR64. The drought-tolerance capacity of Deyou4727 can be used in the future as a drought-resistant germplasm for breeding more drought-tolerant hybrid rice varieties.

Keywords: drought tolerance; *DRO1*; deep root; enzyme activity; germplasm



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1. Introduction

Rice (*Oryza sativa* L.) is one of the most widely consumed and produced cereals, serving more than three billion people worldwide [1]. Crop production and providing global food security remains a challenge for the modern world, as food demand increases at a rate even faster than the population growth [2]. The statistics modeling predicts that the requirements for agricultural products to feed the growing population will be two times higher by 2050 [3]. However, such increasing food demand ultimately challenges the sustainable use of China's agricultural water supply [4], leading to an urgent need for the modification in our agricultural practices.

Rice is considered a water-inefficient crop, and it is cultivated mainly under plentiful water ecotypes (using approximately 30% of the total freshwater available for agriculture) [5]. Drought stress during the grain filling stage is one of the restrictive factors in rice production, and this is expected to upsurge under the current situation of climate change [6,7]. The increasing demand for freshwater in the agricultural sector threatens global rice production due to insubstantial water resources [5]. At the same time, drought

stress due to climate change is currently a major limitation in rice production, which will affect approximately 23 million ha of rainfed rice worldwide [8]. The unusual weather patterns result in climate change that will cause more regular or severe drought stress events in the near future.

The vulnerability of rice plants to drought stress is well known during various developmental stages [9]. Many agricultural researchers are now looking to a set of natural practices, known as sustainable intensification [10], along with some modern molecular approaches, to overcome food security issues [11]. Among different molecular approaches, the development of genetically modified rice varieties with tolerance to drought stress offers an economically viable and sustainable option to improve rice productivity [12], but progress is slow due to the lack of suitable germplasm donors exhibiting a high level of drought tolerance [13]. The genes and transgenic approaches for drought tolerance rely heavily on hybrid rice, which is one of the most consumed and greatest innovations in the world, contributing handsomely to global food security over the past 30 years [1,14]. The responses of rice plants to drought are believed to be complex, involving various physiological, biochemical, and molecular changes [15–17]. Hybrid rice plants can develop a deep and thick root system to improve the hydraulic properties of rice plants under drought stress [18]. These features greatly enhance the drought tolerance capacity of rice by absorbing water stored in the deep soil and yielding more grain [19,20]. Genes controlling root architecture play an important role in soil moisture absorption; therefore, significant effort has been made to develop molecular breeding strategies for improving drought tolerance [21]. *DRO1* is among various key genes which provide drought tolerance in rice via the regulation of root traits and transpiration efficiency [22], and *DRO1* expression is regulated by auxin via auxin response factor (*OsARF*) transcription factors [23]. Similarly, *OSPIN* family genes are reported to be involved in root hair improvement and the initiation of the crown and lateral roots in rice [24]. Thus, to understand drought tolerance in hybrid rice, it is vital to study the relative expression of these genes.

Over the century, thousands of studies have been conducted to screen drought-tolerant germplasm in various corners of the world. Consequently, only a few drought-tolerant varieties were recognized by the International Rice Research Institute (IRRI) in the 1960s [25]. For example, in IR64, the three QTL lines (IR 87707-445-B-B, IR 87707-446-B-B, and IR 87707-182-B-B) were developed by IRR1, possessing great drought tolerance and yielding 0.5 to 1.8 t ha⁻¹ under different severities of drought [26]. Apart from IR64, the selection and breeding of such drought-tolerant rice varieties saw no significant development during this period.

With the exception of IR64, the development of deep-root hybrid rice has become one of the better options for enhancing the drought tolerance of rice plants grown in the Sichuan Basin area, reducing the yield loss of 5.08–34.3% from drought disaster [27–30]. Hybrid rice provides a great opportunity for achieving high yield potential under favorable conditions, but the performance of hybrids under drought stress has not yet been fully evaluated [31]. The hybrid rice is considered genetically diverse, and the genetic basis of hybrid rice “heterosis” is the most important tool in the study of different agronomically important traits and loci. Among various drought-tolerant hybrid rice varieties, Deyou4727 is three-line hybrid rice (a cross between the maintainer line Dexiang074B and the restorer line Chenghui727). Previous studies showed that Deyou4727 has a better capacity to survive drought stress [30]. However, factors that provide better drought tolerance and the important drought tolerance qualities inherited from each parent have not yet been fully studied and understood. Consequently, there is no proper mechanism and comparative understanding related to drought tolerance in IR64 and Deyou4727. That is why, in this study, we aimed (1) to evaluate IR64 and Deyou4727 yield and morphological traits, along with factors responsible for surviving drought stress, under irrigation and drought conditions; (2) to evaluate the root growth, gene expression, and oxidative enzyme activity involved in drought stress; (3) to ascertain the parent (Dexiang074B or Chenghui727) from which the drought-tolerance capacity of Deyou4727 is inherited, through the study of the

ORF regions of the related genes, and (4) to suggest a suitable new germplasm donor with a high level of drought tolerance when compared to IR64.

2. Materials and Methods

2.1. Site Description

We conducted experiments on the campus of the Southwest University of Science and Technology (SWUST), Mianyang (31.55° N, 104.68° E), Sichuan, China, which is a subtropical monsoon climatic area, with an annual temperature is 16–19.2 °C; the frost-free period is 253–301 days, the annual precipitation is 750–1500 mm, and the annual sunlight is 1300–1328 h. For the current experiment, an outbuilding and a particularly designed pot system (China National Invention Patent, ZL201610221861.1) were used to provide drought conditions, according to the experiment. The rice seedlings were grown and transplanted during the rice-growing season (April to September). The soil used in this study was typical sandy loam, which has 64.2% sand, 21% silt, and 15% clay. The organic matter of the soil was 0.25 g/kg, the total phosphorus was 0.20 g/kg, the total nitrogen was 1.18 g/kg, the total potassium was 15.61 g/kg, the perennial moisture content of the soil was 9.2–0.9%, and the soil bulk density was 1.2 g/cm³, respectively. In order to reduce the nutrition deficiency stress on the rice growth during irrigation and drought stress, we mixed 14 g/kg nitrogen, 6 g/kg phosphorus, and 4.2 g/kg potassium into the soil for each pot.

2.2. Experimental Design

Deyou4727 three-line hybrid was selected as the typical drought tolerant deep-root rice [32] bred by the Sichuan Academy of Agricultural Sciences (Deyang, China), and the shallow-root rice selected was IR64 [30], bred by the International Rice Research Institute (IRRI) the Philippine Seed Board with the designation “IR64” in 1985. To confirm the drought-tolerance trait inheritance, Chenghui727 (restorer line) and Dexiang074B (maintainer line) were selected as the parents of Deyou4727 for this experiment, bred by the Sichuan Academy of Agricultural Sciences (Deyang, China). We followed a completely randomized block design (RCBD) to grow 4 rice varieties, with 3 replications of each, in 12 plots under an outbuilding. A sufficient number of seeds (approximately 2×) of each rice variety was seeded in each assigned plot in the outbuilding of the rice research institute of the SWUST. The unhealthy rice seedlings were removed manually during the three-leaf stage, while the healthy rice seedlings were retained for each rice type in their respective plots. The water irrigation was controlled manually by providing drought or irrigation conditions, respectively.

2.3. Samples and Measurements

2.3.1. Root Distribution and Morphology

For the root development and morphology study, we cut inner pots into three parts: the upper soil layer (0–10 cm), the middle soil layer (10–30 cm), and the deep soil layer (30–50 cm). Roots were carefully rinsed with distilled water, the root length was measured using a standard ruler, and the root number was counted for each part. The rotten (broken or decayed) and less than 1 cm in length roots were excluded from the total root number and root length. The roots of all four rice varieties were sampled at the seedling stage to observe the drought stress on the elongation of the root epidermal cells, first using a two-dimensional study (plate pot system), and then the root morphology was analyzed in three dimensions using a CT scanning machine. For the study of the root epidermal cells, thin, fresh root segments were cut and fixed in FAA (formalin: acetic acid: ethanol) solution on a clean glass slide. The fixed roots were then processed with the staining procedures described by Vohník et al. [33]. The root epidermal cells were observed after clarifying the root intersection with 10% KOH at 121 °C for 15 min, then acidifying and staining were completed with 0.5% HCl for 20 s, and 0.05% trypan blue for 5 min. The excess stain was removed and the epidermal cells structure was assessed using a Nikon Eclipse E600 microscope.

2.3.2. Antioxidant Enzyme Activity

The analysis of SOD, POD, and CAT were separately performed for leaves and roots. The freshly cut root and leaf samples were wrapped in aluminum foil and frozen in liquid nitrogen. The frozen samples were crushed with the help of a pestle and mortar, and about 0.5 g of the powdered sample were mixed with 5 mL 50 mM of sodium phosphate buffer (pH 7.8); the mixture was $12,000\times$ g for 15 min and the supernatant was collected for SOD, POD, and CAT estimation.

The SOD activity was determined according to the procedure of Han et al. [34]. In detail, 2.8 mL 50 mM sodium phosphate buffer (pH 7.8), 130 Mm methionine, 0.1 mM ethylenediaminetetraacetic acid (EDTA), 0.75 mM Nitro blue tetrazolium (NBT), 20 μ M riboflavin, and 50 μ L of the enzyme extract (supernatant) or 50 μ L distill water (blank) were used. Finally, the riboflavin was added in the tube mixture to start the reaction. The mixture was vortexed and incubated under constant light (4000 lx) for 20 min. The reaction was stopped in the dark, and absorbance was measured at 560 nm. SOD activity was expressed as nanogram/mL [35]. For peroxidase (POD), the reaction mixture contained 2.5 mL of 50 mM sodium phosphate buffer (pH 7), 1 mL of 1% H₂O₂, 1 mL of 1% guaiacol, and the enzyme extract (supernatant). The changes in absorbance were measured at 420 nm for 1 min. The activity was expressed in units, where one unit represented an increase in absorbance of one in one minute [36]. The CAT activity was measured according to the method of Aebi, [37]. In detailed, we mixed 100 μ L enzyme source into 6 mL 50 mM sodium phosphate buffer (pH 7.8) and 20 mM H₂O₂. The enzyme activity was recorded at a 10 s interval for 4 min at 240 nm. The optimal activity was measured and defined as the decline of absorbance at 240 nm 0.1 unit per minute. The CAT was expressed as U g⁻¹ FW min⁻¹.

2.3.3. Gene Expression

After germination, equal seedlings of Deyou4727, IR64, Chenghui727, and Dexiang074B were cultured in IRR1 solution. Seven days later, the roots of the seedling were collected for quantitative real-time PCR (qRT-PCR). The quantitative real-time PCR (qPCR) was performed using the SYBR Premix Ex Taq (Takara, Dalian, China) and the CFX96TM Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). The PCR mixture was composed of 5 μ L SYBR Premix Ex Taq, cDNA corresponding to 30 ng RNA, 0.3 μ L of each F and R primer (10 mM), and PCR-grade deionized distill water up to the final volume of 10 μ L. The incubation temperatures of the reaction were denaturation (at a cycle of 95 °C for 3 min), amplification (40 cycles at 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s), elongation (72 °C for 3 min), and melting curve analysis (65 to 95 °C) [38]. *ACTIN1* gene was used as an internal control. The primer sequences for *DRO1*, *OsPIN1b*, *OsPIN9*, and *OsARF23* are available in Supplementary Table S1.

2.3.4. Deeper Rooting 1 (*DRO1*) Gene Sequencing

The *DRO1* gene sequencing was conducted according to our previous study [30]. In detail, fresh leaf tissues were sampled from each rice variety and immediately transferred to liquid nitrogen for freezing. The DNA was extracted from frozen leaf samples using the cetyltrimethylammonium bromide (CTAB) method [39]. The *DRO1* gene sequence and primer details are shown in Table S1. The amplified samples were sent to TsingKe Biological Technology Company (Chengdu, China) for *DRO1* gene sequencing.

2.3.5. Tools and Instruments

The rice cultivating pot system and the plate pot system were used as tools to check the root distribution in different soil layers and root morphology in two dimensions. The CT machine (Siemens Healthineers) was used to obtain the root morphology in three dimensions. The imaging of the cell surface and cell outline in plant tissues was completed using a compound microscope.

2.4. Statistical Methods

The variance among different rice varieties were calculated using one-way ANOVA analysis in SPSS (Version 20, Chicago, IL, USA), using the least significant difference statistic (LSD = 0.05). Origin 19.0 and Excel 2019 statistical software were used for data visualization. Each treatment value is expressed as mean \pm standard deviation (SD) of 3–6 biological replicates. We performed principal component analysis (PCA) to assess potential differences between rice varieties by using the FactoMineR-package, and Pearson's correlation coefficient was obtained done using ggcorrplot in R version 4.0.3 [40,41].

3. Results

3.1. Response of Yield and Yield Components under Irrigation and Drought Conditions

Compared to irrigation treatments, the yields of both rice varieties (IR64 and Deyou4727) were significantly reduced under drought conditions. More specifically, Deyou4727 and IR64 showed 33 and 25 (g) grain yield per plant (GY) under irrigation treatments. The GY of both rice varieties reduced under drought conditions, which was significantly lower in IR64, (20 g grain yield per plant), as compared to Deyou4727 (30 g grain yield per plant). Similarly, the number of maximum tillers (MT) was high in the irrigation treatments for IR64 (23.3) as compared to Deyou4727 (17.7), but the MT of IR64 decreased (20.3) under drought treatment, but not for Deyou4727 (17.2). Unlike MT, the panicle length (PL) was high in Deyou4727 (24.2) as compared to IR64 (23.8) under irrigation treatment, which reduced significantly ($p < 0.05$) in Deyou4727 (21), but not in IR64 (22.4) (Figure S1). Similarly, effective panicle (EP) was high in IR64 at irrigation (15.3) and drought (13) treatments as compared to Deyou4727 (12.3–10.7) (Table 1). Unlike PL and EP, spikelet number (SN) was significantly high ($p < 0.05$) in Deyou4727 under both irrigation (148) and drought (98.3) treatments, while IR64 exhibited low values of SN under irrigation (85.7) and drought (55.7) treatments, respectively. The seed setting rate percentage (SSR%) showed no significant difference among both varieties under irrigation and drought treatment; however, both showed high SSR% under irrigation (86–87%) and low SSR% under drought (75%) treatment.

Table 1. The response of yield and the yield components of IR64 and Deyou4727 under irrigation and drought conditions.

	Varieties	Grain Yield (g/per Plant)	MT	PL	EP	SN	SSR (%)
Irrigation	IR64	25 \pm 1.50 b	23.3 \pm 1.11 a	23.8 \pm 1.02 a	15.3 \pm 1.41 a	85.7 \pm 1.21 b	86.7 \pm 2.29 a
	Deyou4727	33 \pm 1.58 a	17.7 \pm 1.16 b	24.2 \pm 1.08 a	12.3 \pm 1.26 b	148.0 \pm 2.17 a	86.3 \pm 1.51 a
Drought	IR64	20 \pm 1.16 b	20.3 \pm 1.28 a	22.4 \pm 1.45 a	13.0 \pm 1.03 a	55.7 \pm 1.59 b	75.1 \pm 1.11 a
	Deyou4727	30 \pm 1.62 a	17.2 \pm 1.29 b	21.1 \pm 1.15 a	10.7 \pm 0.97 b	98.3 \pm 1.17 a	75.3 \pm 1.27 a

MT, Number of maximum tillers; PL, Panicle length; EP, Number of effective panicles; SN, Spikelet number per panicle; SRR, Seed-setting rate. Values are the mean \pm standard deviation of 3–6 replicates, and values followed by different letters within a row indicate difference ($p < 0.05$) among different parameters.

3.2. Root Distribution of Deyou4727 in 0–50 cm Soil

Under irrigation treatments, the total root length (TRL) of IR64 was mainly distributed in the 0–10 cm soil layer (4005), which accounted for more than 80% of the TRL in the 0–50 cm soil layer. IR64 showed significantly ($p < 0.05$) low TRL in 10–30 and 30–50 cm of soil, with only 15% of TRL under irrigation treatment, respectively. Unlike IR64, we found low TRL (1616) in 0–10 cm of soil for Deyou4727 under irrigation treatment, which is a significant ($p < 0.001$) difference. However, compared to IR64, the TRL was significantly high in 10–30 (1725) and 30–50 (721) cm of soil for Deyou4727 (Figure 1A). We found that, under drought stress, IR64 produced high TRL (4545) as compared to Deyou4727 (2894) in the 0–10 cm soil layer, respectively, which is consistent with irrigation treatment. Unlike irrigation treatment, under drought stress, TRL dropped significantly for IR64 (719) in the 10–30 cm soil layer, while TRL decreased slightly for Deyou4727 (2340) at the same soil

layer. The trend of TRL followed a similar pattern at 30–50 cm soil as well, in which IR64 only showed 247 TRL and Deyou4727 showed 1365 TRL, respectively (Figure 1B).

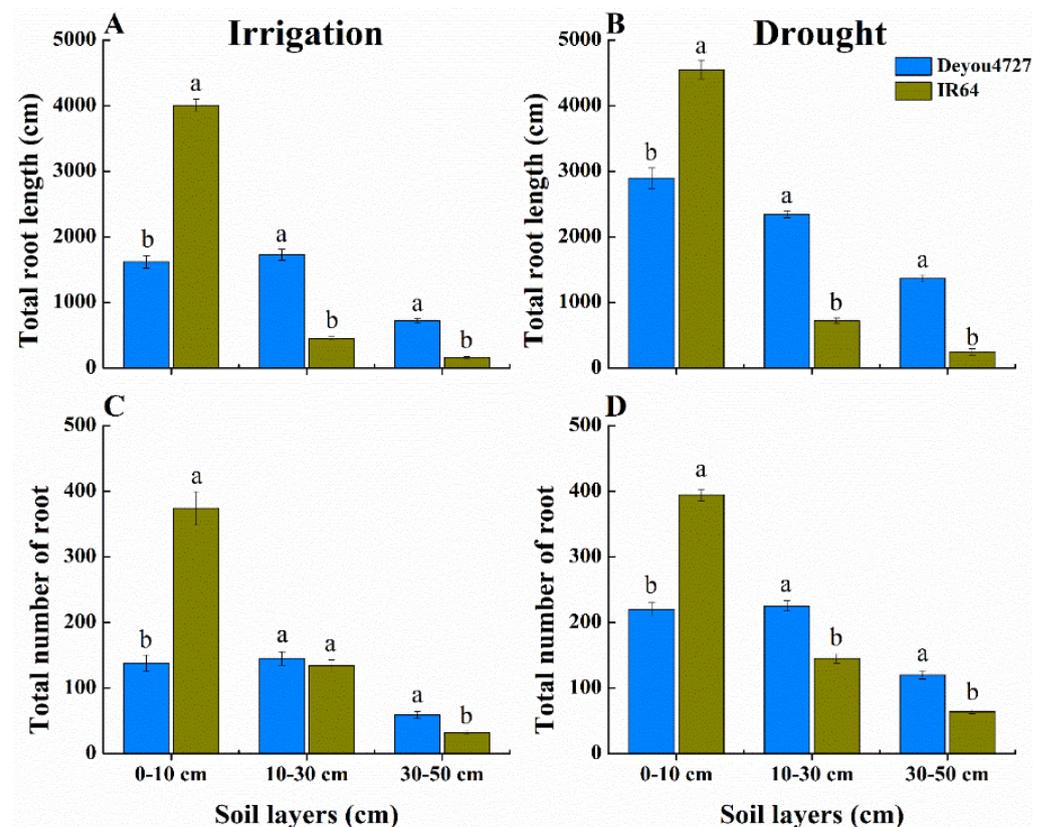


Figure 1. The comparison of the total root length (A,B) and total number of roots (C,D) among different soil layers of Deyou4727 and IR64 under irrigation and drought conditions. The values in each graph represent the Mean \pm SE (standard error) of 3–6 replicates, and lowercase letters indicate significant differences between varieties and treatments, at $p < 0.05$.

The total number of roots (TNR) was at the maximum in the 0–10 cm soil layer under irrigation treatment for IR64 (374), while Deyou4727 showed significantly low (138) TNR. As the layer of soil deepened (10–30 cm), the TNR values decreased significantly for IR64 (134), but not for Deyou4727 (145). Similarly, at the deepest soil layer (30–50 cm), the TRN values were lowest for IR64 (32), while Deyou4727 showed significantly ($p < 0.05$) high (59) TRN values (Figure 1C). Both rice types showed significantly higher TRN at drought stress. The shallow layer soil (0–10 cm) showed the most roots for IR64 (394), while Deyou4727 had low (220) TRN at the same soil layer. The rice type IR64 (145) had low TRN in 10–30 cm soil as compared to Deyou4727 (225), which was consistent in the 30–50 cm soil level as well (Figure 1D).

3.3. Root Distribution and Morphology Comparison with Parents

We found that Deyou4727 produced more and longer roots than Dexiang074B and Chenghui727 in the 0–10 cm soil layer, 10–30 cm soil layer, or 30–50 cm soil layer (Figure 2A). The difference of TRN between Dexiang074B and Chenghui727 was not consequential in the 0–10 cm soil and 10–30 cm soil layers, but was significant in the 30–50 cm soil layer (Figure 2B). More specifically, at the shallow layer of soil (0–10 cm), we found a significant difference of TRL for Dexiang074B (1281), Chenghui727 (1504), and Deyou4727 (1616). As the soil layer increased to 10–30 cm of soil, the TRL values were highest for Deyou4727 (1724), followed by Chenghui727 (1539), and Dexiang074B (1477). At the deepest soil layer,

the TRL values were high for Deyou4727 (721) and Dexiang074B (675), but reduced for Chenghui727 (290).

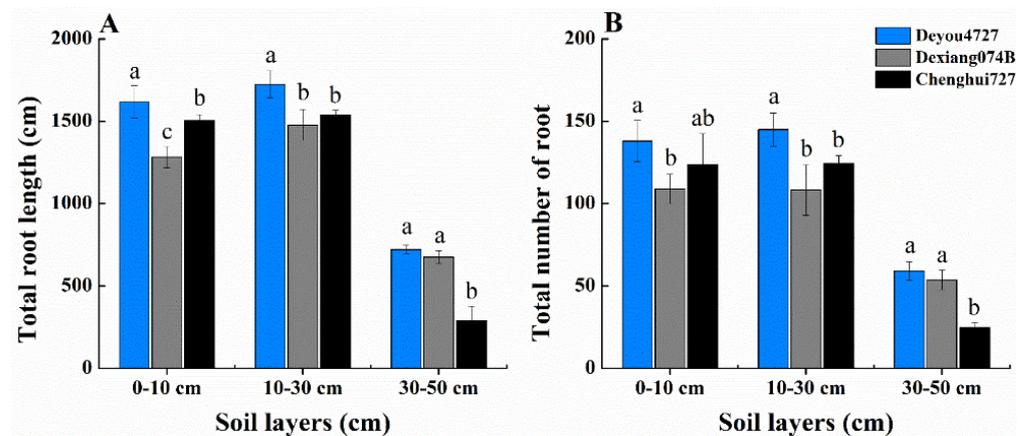


Figure 2. The root distribution of the total root length (A) and total number of roots (B) in different soil layers of Deyou4727 and its parents. The values in each graph represent the Mean ± SE (standard error) of 3–6 replicates, and lowercase letters indicate significant differences between varieties and treatments, at $p < 0.05$.

Further, we analyzed the root morphology using CT-Scanning and the results showed that Deyou4727 and Dexiang074B developed more vertical roots than IR64 during the seedling stage (Figure 3). We also found that the number of vertical roots in Dexiang074B was not significantly different from Deyou4727. At the same screening, the Chenghui727 root morphology showed a huge difference between Deyou4727 and Dexiang074B.



Figure 3. The root morphology of IR64, Deyou4727, and its parents in two dimensions (A) and three dimensions (B).

To explain the root phenotype of IR64, Deyou4727, Dexiang074B, and Chenghui727 under drought stress, we analyzed the shape of the epidermal cells in the root differentiation zone using 7-day-old seedlings (Figure 4A). In Deyou4727 and Dexiang074B, the epidermal cells were longer and more tabulated than those of IR64 and Chenghui727 (Figure 4B–I). The long and tabulated epidermal cell formation defines Deyou4727 and Dexiang074B as distinctive and may contribute to root protection under drought conditions.

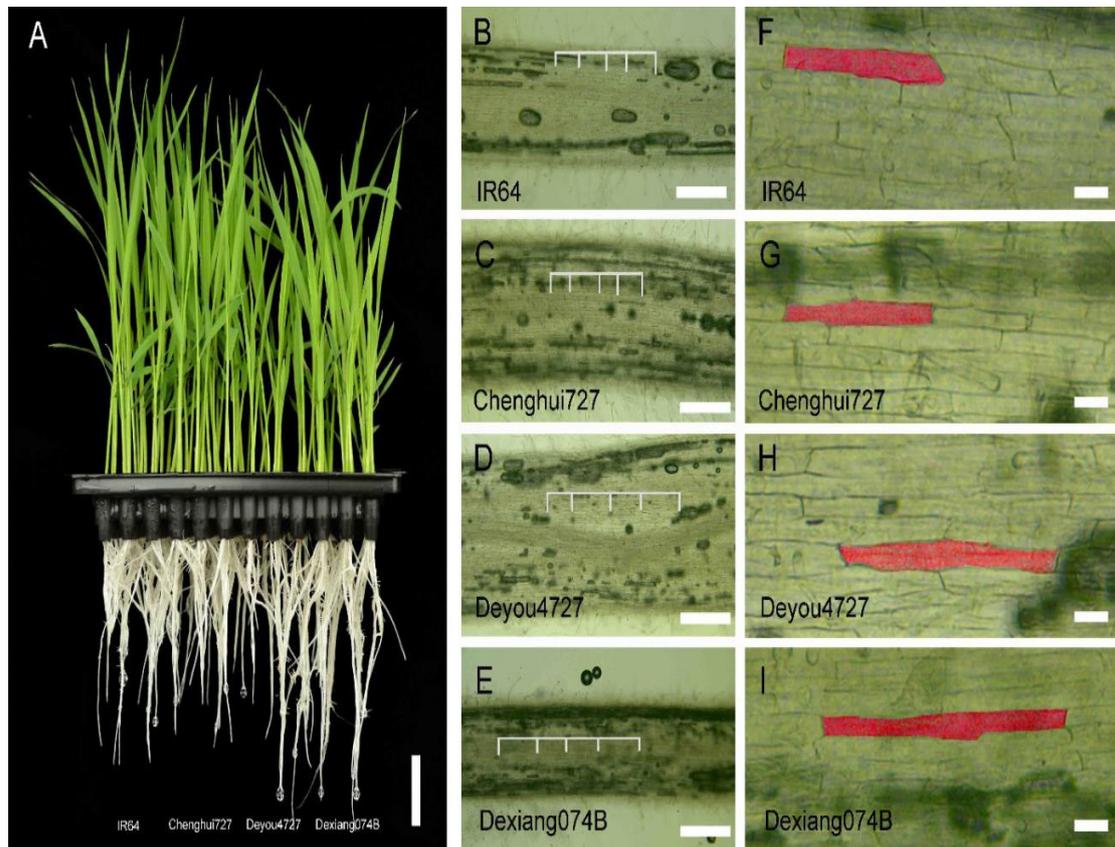


Figure 4. The morphology of epidermal cells in IR64, Chenghui727, Deyou4727, and Dexiang074B. (A) The 7-day-old seedlings of IR64, Chenghui727, Deyou4727, and Dexiang074B (bar = 2 cm). (B–E) Photos of the differentiation zone in IR64 (B), Chenghui727 (C), Deyou4727 (D), and Dexiang074B (E) roots, and the length of four cells are marked. (F–I) The shape of the typical epidermal cell in IR64 (F), Chenghui727 (G), Deyou4727 (H), and Dexiang074B (I). Scale bars: 2 cm in (A); 100 μ m in (B–E); 10 μ m in (F–I).

3.4. The Activity of the Antioxidant Enzyme

To assess the antioxidant enzymes under irrigation and stress conditions, we analyzed the activity of catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD) in IR64, Deyou4727, Dexiang074B, and Chenghui727 in the leaves and roots, respectively (Table 2). We found that under irrigation treatment, all rice types showed low CAT activity in leaves and roots. The rice type IR64 (635, 557) and Dexiang074B (638, 562) showed no significant difference in CAT activity, while Chenghui727 (557, 435) and Deyou4727 (560, 452) showed low CAT enzyme activity in leaves and roots, respectively. The POD enzyme activity in the leaf was high for Dexiang074B (10) in roots and leaves, while IR64 (5), Chenghui727 (6), and Deyou4727 (7) showed no significant difference of POD in the leaves. The POD enzyme activity varied greatly in the roots of IR64, which showed maximum activity of (14), while Deyou4727 and Chenghui727 showed no significant difference. We found the highest SOD activity in the IR64 leaves (67), followed by Deyou4727 (57), and Chenghui727 (57) (no significance difference), and the lowest values were found in Dexiang074B (40). The SOD values in the roots were high for Dexiang074B (53), followed by IR64 (49), Deyou4727 (41), and Chenghui727 (40).

Table 2. The effects of drought stress on antioxidant enzyme activity in different rice varieties.

Treatments	Varieties	CAT		POD		SOD	
		Leaf	Root	Leaf	Root	Leaf	Root
Irrigation	IR64	635 ± 17 a	554 ± 17 a	5 ± 1.2 b	14 ± 1.3 a	67 ± 4.6 a	49 ± 3.8 a
	Deyou4727	560 ± 16 b	452 ± 14 b	7 ± 1.3 b	9 ± 1.1 b	57 ± 3.4 b	41 ± 4.6 b
	Chenghui727	557 ± 15 b	435 ± 12 b	6 ± 1.1 b	8 ± 1.3 b	40 ± 3.5 c	40 ± 4.2 b
	Dexiang074 B	638 ± 18 a	562 ± 15 a	10 ± 1.2 a	10 ± 1.1 ab	57 ± 3.3 b	53 ± 3.6 a
Drought	IR64	756 ± 16 b	894 ± 19 b	17 ± 1.1 b	29 ± 3.2 b	57 ± 4.3 b	75 ± 4.2 a
	Deyou4727	800 ± 17 a	977 ± 16 a	20 ± 1.8 a	33 ± 3.4 a	70 ± 5.2 a	74 ± 4.3 a
	Chenghui727	787 ± 14 ab	675 ± 15 c	18 ± 2.3 ab	31 ± 3.2 ab	78 ± 5.3 a	74 ± 4.6 a
	Dexiang074 B	820 ± 15 a	987 ± 18 a	22 ± 2.9 a	36 ± 3.2 a	61 ± 4.6 b	75 ± 4.7 a

SOD, Superoxide dismutase; CAT, Catalase; POD, Peroxidase. Values are the mean ± standard deviation of 3–6 replicates, and values followed by different letters within a row indicate difference ($p < 0.05$) among different parameters.

The antioxidant enzymes CAT, POD, and SOD increased sufficiently in all rice types under drought conditions. The CAT activity in the leaves showed no significant difference in all rice types; however, it varied greatly in the roots. The CAT activity in the roots was high in Dexiang074B (987) and Deyou4727 (977), followed by IR64 (894) and Chenghui727 (675). For the drought stress-induced changes in the POD of all rice types; however, Chenghui727, Dexiang074B, and Deyou4727 showed no significant difference of POD values in leaves, while IR64 (17) exhibited a significantly low POD activity. Unlike for leaves, the root POD values were significantly high in drought stress for all rice types, and there was no significant difference between IR64, Chenghui727, Dexiang074B, and Deyou4727. The SOD values in the leaves were high for Chenghui727 (78) and Deyou4727 (70). The rice type IR64 and Dexiang074B showed significantly low SOD activity (57 and 61). The SOD activity of the roots showed no significant difference among all rice types under drought conditions.

3.5. Gene Expression of *OsPIN9*, *OsPIN1b*, *OsARF23* and *DRO1*

We sequenced the different gene expressions under drought treatments for IR64, Deyou4727, Dexiang074B, and Chenghui727. We found no significant difference in the relative expression of *OsPIN9* and *OsARF23* in IR64, Dexiang074B, Deyou4727, and Chenghui727 (Figure 5A,C). However, the *OsPIN1b* gene showed significantly high relative expression in Dexiang074B and Deyou4727. The lowest relative expression of the *OsPIN1b* gene was found in IR64 and Chenghui727 (Figure 5B). Similarly, the relative expression of the *DRO1* gene was high in Deyou4727, Dexiang074B, and Chenghui727, while its values were reduced in IR64 (Figure 5D).

We also looked into the *DRO1* gene open reading frame region (ORF), and we found that Deyou4727 and its parent lines shared the same open reading frame (ORF), and no difference in UTR, 5'UTR, EXON3, and 3'UTR were found (Figure S2B). However, in EXON 4, we found that IR64 showed a difference in adenine. The rice types Deyou4727, Dexiang074B, and Chenghui727 exhibited adenine at EXON 4, while IR64 lacked adenine at EXON 4 (Table 3). The ORF studies also disclosed that differences in codons on type-I and type-II mRNA caused the translation of different amino acids, such as aspartic acid in the case of Deyou4727, Dexiang074B, and Chenghui727, and valine in the case of IR64.

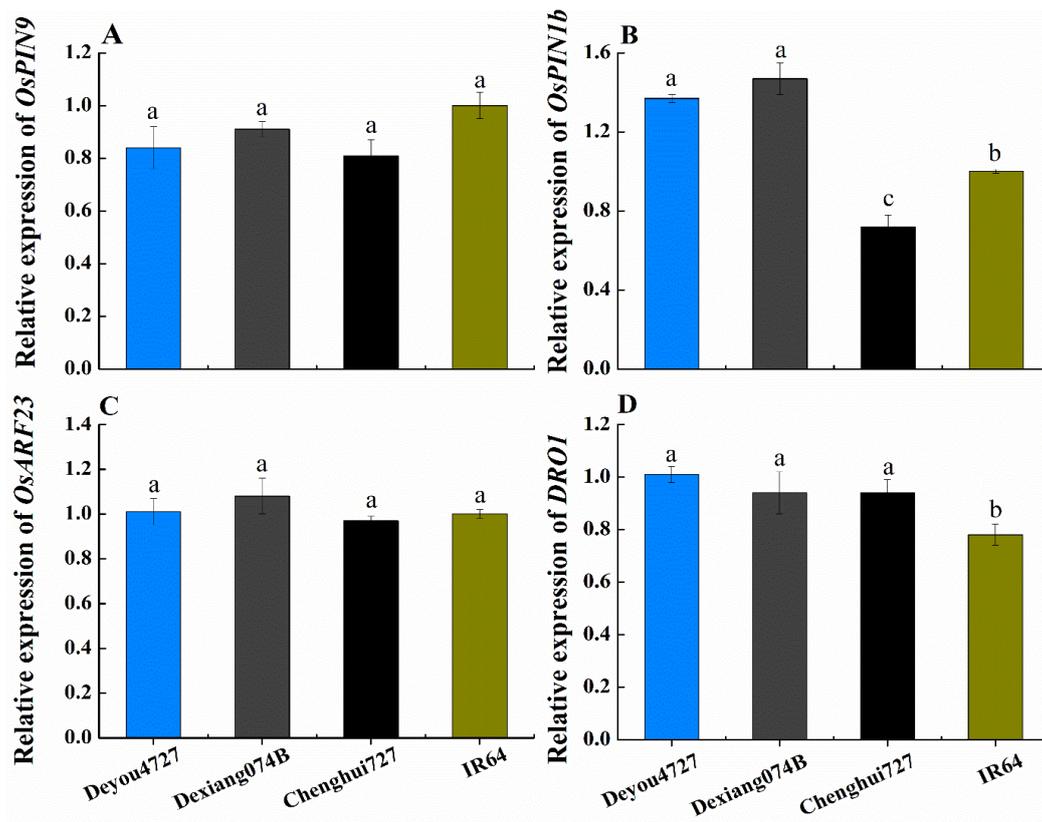


Figure 5. The expression of *OsPIN9* (A), *OsPIN1b* (B), *OsARF23* (C), and *DRO1* (D) in Deyou4727, Dexiang074B, Chenghui727, and IR64. The values in each graph represent the Mean \pm SE (standard error) of three replicates, and lowercase letters indicate significant differences between varieties and treatments, at $p < 0.05$.

Table 3. The *DRO1* sequence of Deyou4727, its parents, and IR64.

Material	UTR	5'UTR	EXON 3	EXON 3	EXON4	EXON4	3'UTR
cDNA	27	150	617	752	943	962	1133–1134
gDNA	27	150	2096	2231	2513	2532	2909–2910
Seq (+)	A/G	C/T	T/C	A/C	A/-	C/A	CT/-
IR64	G	T	C	A	-	C	-
Deyou4727	A	T	C	C	A	C	-
Chenghui727	A	T	C	C	A	C	-
Dexiang074B	A	T	C	C	A	C	-

3.6. Multivariate and Pearson's Correlation Coefficient Analysis of Root Traits, Responsible Genes, and Enzymatic System

We performed multivariate analyses through PCA to better understand the relationships involved in drought stress across the different varieties (Figure 6). The variability of different root traits, responsible genes, and enzymes was explained by PC1 and PC2. PC1 explained 42.3% of the variation and PC2 explained 27.6% of the variation. We found that, under drought stress, PC1 was positively associated with TRN and TRL (10–50 cm soil), *OsPIN1b*, *DRO1*, POD, and CAT, and negatively associated with TRN, TRL (0–10 cm soil), and SOD. Under the same conditions, PC2 was positively associated with TRN (0–10 cm soil), TRL (0–10 cm soil), SOD, TRN (30–50 cm soil), TRN (10–30 cm soil), CAT, TRL (30–50 cm soil), and *OsPIN1b*, while negatively associated with TRL (10–30 cm soil), POD, and *DRO1*, respectively.

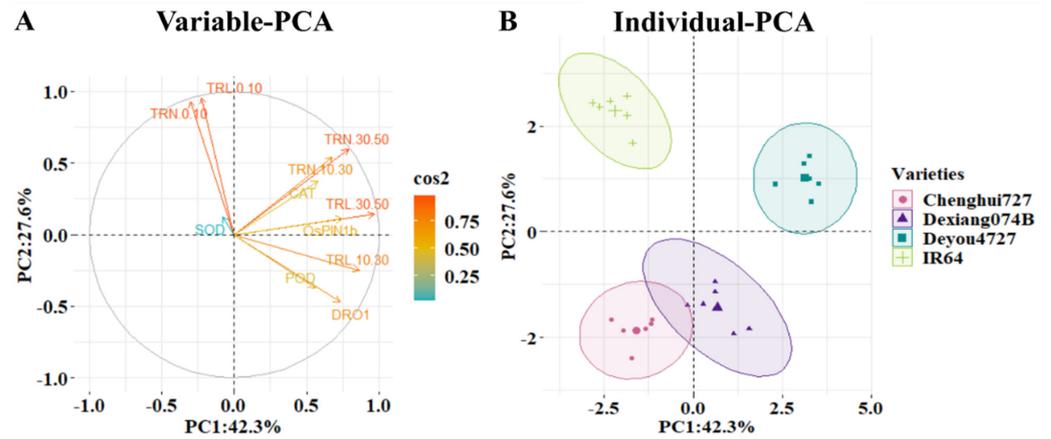


Figure 6. The multivariate principal component analysis (PCA) for IR64, Chenghui727, Dexiang074B, and Deyou4727 considering root traits, responsible genes, and enzymatic systems. TRN = the total number of roots at the top (0–10 cm), middle (10–30 cm), and bottom (30–50 cm); TRL = the total root length at the top (0–10 cm), middle (10–30 cm), and bottom (30–50 cm), and the activity of CAT, SOD, POD, and the expression of the *OsPIN1b* and *DRO1* genes. (A) The loading plot of the physiological and biochemical variables for the first (PC1) and second (PC2) principal components for variables. (B) Sample score of the individuals for IR64, Chenghui727, Dexiang074B, and Deyou4727.

The correlation coefficient for irrigation and drought for plant traits and grain yield (GY) under irrigation and drought were measured (Figure 7). Under irrigation conditions, TRN-M ($r = 0.8$), TRN-B ($r = 0.9$), SN ($r = 1$), TRL-M ($r = 0.9$), TRL-B ($r = 0.9$), and POD-L ($r = 0.9$) showed significant ($p < 0.05$) positive correlation. Similarly, under drought conditions, SN ($r = 1$), TRN-M ($r = 0.9$), TRN-B ($r = 0.9$), TRL-M ($r = 1$), TRL-B ($r = 1$), CAT-L ($r = 0.8$), SOD-L ($r = 1$), POD-L ($r = 1$), and CAT-R ($r = 1$) showed significant ($p < 0.05$) positive correlation. The oxidative enzymes POD, SOD, and CAT are important under drought stress; these indicate that versatile enzymatic system helped plants to survive drought conditions and increase yields.

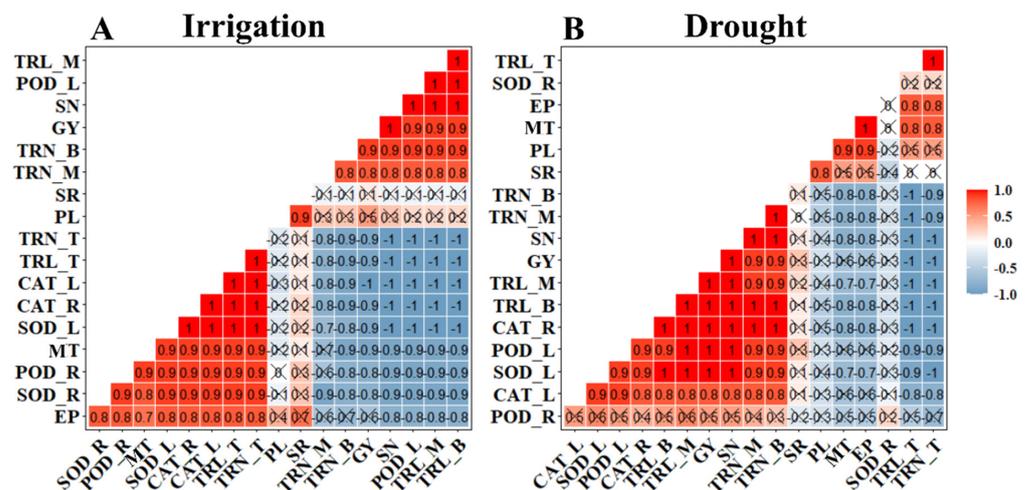


Figure 7. The Pearson’s correlation matrix among traits and oxidative enzymes, under irrigation (A), and under drought (B). The scale bar on the right shows the negative and positive intensity of correlation (1 to -1). GY, Grain yield; MT, Number of maximum tillers; PL, Panicle length; EP, Number of effective panicles; SN, Spikelet number per panicle; SRR, Seed-setting rate; TRN_T, Total number of roots at the top (0–10 cm); TRN_M, Total number of roots at the middle (10–30 cm); TRN_B, Total number of roots at the bottom (30–50 cm); TRL_T, Total number of roots at top (0–10 cm); TRL_M, Total number of roots at the middle (10–30 cm); TRL_B, Total number of roots at the bottom (30–50 cm); SOD_L, Superoxide dismutase in the leaves; SOD_R, Superoxide dismutase in the roots; CAT_L, Catalase in the leaves; CAT_R, Catalase in the roots; POD_L, Peroxidase in the leaves; and POD_R, Peroxidase in the roots.

4. Discussion

Drought and water scarcity are serious limitations for crop production in rain-fed areas [42]. Therefore, food security in the 21st century will be progressively more dependent on the release of new cultivars with improved adaptation to drought conditions [43]. In the past, various strategies have been employed to combat such disasters, such as plant breeding and genetically modified plant adaptation [30]. However, selection for drought tolerance is difficult due to complex genotypes and environmental interactions. In this study, a hybrid rice, Deyou4727, was comparatively analyzed with IR64 over a range of experiments using irrigation and drought stress environments. The Deyou4727 was also compared with its inbred parents (Dexiang074B, and Chenghui727) in order to clarify the drought resistance inheritance. We found that Deyou4727 showed significantly higher grain yield under irrigation and drought treatments as compared to IR64. The hybrid rice Deyou4727 showed a higher spikelet number as compared to IR64, making it suitable for higher grain production under drought conditions. Our findings are consistent with previous studies, in which Villa et al. [31] reported significantly greater grain yield in hybrid rice over inbred rice cultivars during severe drought stress.

The drought tolerance in hybrid crops is directly associated with versatile plant physiological traits. Previous studies have found that proper root architecture is the key factor for rice to resist drought stress [44]. Drought-tolerant varieties usually form a deep and thick root system under drought stress [45,46], which enables the rice to absorb more deep-soil water to supply its growth during the dry season [45,46]. The deep root system also improves the utilization of nutrition in the deep soil by promoting the transportation of water from deep soil to the plant, directly improving the performance of rice, as seen in characteristics such as the development of more tillers and more effective panicles during the dry season [47]. In our study, we found the total root number (TRN), total root length (TRL), and proportion of total roots of Deyou4727 in 30–50 cm soil were higher than for IR64 under drought conditions. Similar findings were reported in our previous study, in which, by field study, we selected the most suitable rice plants under drought stress [30]. We found Deyou4727 was one of the favorable hybrid rice cultivars to be grown in low water conditions; it exhibited a deep rooting system and high resistance to drought stress. The new hybrid rice cultivars show promising values for combatting drought issues by producing deep roots. Kim et al. [45] reported that an appropriate root structure in the soil improved the sustainability of rice production in a seasonal drought area by using deep-soil water effectively. Similar findings were reported by Lynch et al. [48], who showed an elevation in the yield of cereal crops grown under irrigation and drought by altering the root structure, as this improves their ability to access deep-soil resources. In the case of our study as well, water absorbed from the deep soil enabled Deyou4727 to survive drought stress, providing more nutrition for its growth during dry seasons, and ultimately producing a greater yield than IR64 under drought-stress treatments. The Pearson's correlation coefficient results also showed that root number, root length in middle and bottom layer, and oxidative enzymes were major traits for an increase in grain yield under drought conditions [49].

Besides the improvement of root structure, increased antioxidant enzymatic defense is another important way for plants to survive drought stress by protecting rice from oxidative damage [50]. The enzymatic antioxidant system that regulates reactive oxygen species (ROS) and redox homeostasis includes superoxide dismutase (SOD) [51], peroxidase (POD), and catalase (CAT) [52]. High antioxidant enzyme activity has also been observed in drought-resistant plants from many genera and species [53,54]. The first enzyme in the antioxidant pathway is SOD [55], which removes superoxide radicals by catalyzing their dismutation, one $O_2^{\bullet-}$ being reduced to H_2O_2 and another oxidized to O_2 [56]. In our study, we found that IR64 exhibited significantly low SOD in the leaves when compared to Deyou4727, Dexiang074B, and Chenghui727, while there was no significant difference in the root SOD of all four tested rice types. We also found no significant difference of the CAT enzyme in the leaves under drought stress; however, the CAT and POD enzymes in

were high in the roots of Deyou4727 and Dexiang074B, as compared to IR64. The obtained results indicated that Deyou4727 controlled the damage from drought oxidative stress by using an enzymatic antioxidant defense system.

The advancement of molecular biological tools and functional genomics has facilitated significant progress in identifying some aspects of the drought response in plants [57]. The differing capacity of hybrid rice varieties to adapt to drought stress is associated with different gene expression patterns [58]; therefore, the mechanisms by which drought-responsive gene expression is regulated is of great importance. Previous studies have reported that auxin response factors (*ARF*) and auxin transport (*OsPIN*) family genes are involved in rice seminal root development [59]; however, there was no clear study of its role in drought stress. That is why, in this study, we screened two *OsPIN* families (*OsPIN9* and *OsPIN1b*) and one *OsARF23* gene to check their relative expression in all four rice varieties under drought conditions. We found no significant difference between the expression of the *OsPIN9* and *OsARD23* gene among all varieties; however, *OsPIN1b* was highly expressed in Deyou4727 and Dexiang074B, as compared to IR64.

Similarly, the deeper rooting 1 (*Dro1*) gene is one of the QTLs that significantly contributes to root growth angle, assisting in the deeper rooting of rice plants. In our study, we found that the hybrid rice variety Deyou4727 showed the highest *DRO1* gene expression, followed by Dexiang074B and Chenghui727, while IR64 showed a significantly low expression of the *DRO1* gene under drought stress. The expression of *DRO1* was positively related to total root number and total root length development in Deyou4727 and Dexiang074B. This high expression resulted in deeper root formation. These genotypes possessing the deeper-rooting QTLs can be taken as donor lines to be used in marker-assisted breeding programs.

5. Conclusions

Immense root growth, favorable distribution angle, and extended root length are perfect combinations to overcome seasonal drought and can assist in the development of deep roots in Deyou4727 and Dexiang074B. The deep root development and higher antioxidant enzyme activity of Deyou4727 provide good drought-tolerant capacity and growth in the deep-soil layer. Dexiang074B, acting as the parents of Deyou4727, provided higher drought-tolerant capacity through passing along the *DRO1* and *OsPIN1b* genes. The functional validation of *DRO1* and *OsPIN1b* using reverse genetics approaches, such as genome editing, could provide an important genetic resource for the future breeding of drought tolerant rice.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy12030752/s1>—Table S1. The primer sequences used for qRT-PCR verification; Figure S1. The impact of drought stress during the tiller growth of Deyou4727 (A), and IR64 (B); Figure S2. The open reading frame structure of the *DRO1* gene in Deyou4727, its parents, and IR64.

Author Contributions: X.W. and Y.H. designed and supervised the study; Y.Z. and G.Y. collected the samples; F.N., S.S. and J.Q. conducted the laboratory experiments, completed the writing—original draft preparation, and the formal analysis; S.S., A.R.K. and K.J. completed the writing—review and editing; Y.P. and A.R.K. conducted the bioinformatics and statistical analyses. All authors have read and agreed to the published version of the manuscript.

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