agriculture

MDPI

Article

Overexpressing *OsPYL/RCAR7* Improves Drought Tolerance of Maize Seedlings by Reducing Stomatal Conductance

Joon Ki Hong, Yeon-Hee Lee †, Beom-Gi Kim, Gang Seob Lee, Hee Jeung Jang, Giha Song, Eun Jung Suh and Sang Ryeol Park *

Gene Engineering Division, National Institute of Agricultural Sciences, Rural Development Administration, 370 Nongsaengmyeong-ro, Jeonju 54874, Republic of Korea

* Correspondence: srpark@korea.kr; Tel.: +82-63-238-4582

† Present address: LaSemilla Co., Ltd., 1447 Pyeongchang-daero, Daehwa-myeon, Pyeongchang-gun 25354, Gangwon-do, Republic of Korea.

Abstract: Drought stress is a serious abiotic factor limiting the quality and yield of maize (*Zea mays*). To produce maize plants with enhanced drought tolerance, we generated transgenic maize plants overexpressing *OsPYL/RCAR7*, encoding an abscisic acid receptor. We crossed the selected lines with maize variety B73 and obtained F1 hybrid seeds. Initial screening suggested that the transgenic lines were more drought tolerant than wild-type plants. Analysis using the DroughtSpotter platform indicated that expressing *OsPYL/RCAR7* enhanced drought resistance in transgenic maize seedlings by reducing water loss. In addition, the stomatal conductance of the leaf surface was 30% lower in *OsPYL/RCAR7*-overexpressing plants than in wild-type ones. After drought treatment, *OsPYL/RCAR7*-overexpressing maize showed a much higher survival rate than the wild type, suggesting that expressing *OsPYL/RCAR7* reduced the negative effects of drought exposure on stomatal conductance and enhanced water use efficiency. Furthermore, the expression levels of drought-tolerance–related abscisic acid–signaling genes *ABP2* and *RAB16A* were higher in the transgenic plants than in the wild type. Taken together, our data indicate that the seedlings of transgenic maize expressing the gene *OsPYL/RCAR7* showed increased tolerance to drought stress, raising the possibility that stress-related genes from monocotyledonous crops could be used as genetic resources to improve the agricultural traits of maize.

Keywords: abiotic factor; crop; drought tolerance; maize; transformation; water loss

check for updates

Citation: Hong, J.K.; Lee, Y.-H.; Kim, B.-G.; Lee, G.S.; Jang, H.J.; Song, G.; Suh, E.J.; Park, S.R. Overexpressing *OsPYL/RCAR7* Improves Drought Tolerance of Maize Seedlings by Reducing Stomatal Conductance. *Agriculture* **2022**, *12*, 2140. https://doi.org/10.3390/agriculture12122140

Academic Editors: Vandana Jaiswal, Vijay Gahlaut and Deepmala Sehgal

Received: 8 November 2022
Accepted: 9 December 2022
Published: 13 December 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Maize (*Zea mays* L.), one of the world's top-three crops (along with rice [*Oryza sativa*] and wheat [*Triticum aestivum*]), has adapted to a wide range of environmental conditions and is grown in a variety of soils and environments worldwide [1,2]. Maize is used for multiple agricultural and industrial purposes, such as a raw material for biofuel and for food and livestock feed [3,4]. The growth, development, and productivity of maize are seriously affected by abiotic stresses such as drought and salt stress [5–7]. Drought stress caused agricultural production losses of about 19% around the world, which amounted to more than USD 17 billion (FAOSTAT 2017, http://www.fao.org/faostat/en/#compare, accessed on 8 October 2022) [8]. Substantial reductions in maize productivity are observed under drought stress. In 2012, the US suffered a 12% decrease in maize production by agricultural drought (USDA, 2014) [9]. In addition, dramatic yield losses are expected by the end of the 21st century, such as 10~20% for maize in Korea (KCCAR2020, http://cc_data/2020/Korean_Climate_Change_Assessment_Report-2020_2.pdf, accessed on 8 October 2022). Thus, it is important to explore the drought-stress-response mechanisms of maize and to enhance the drought tolerance of this crop [10].

Drought stress is one of the most important environmental stresses, as it negatively affects agricultural productivity and the food supply [11,12]. Water deficiency inhibits cell

growth and hinders the development of fruits, leaves, and flowers [13]. Plants respond to drought stress by reducing the effects of the lack of water by decreasing their transpiration rates and increasing the efficiency of water acquisition from the soil [14]. Moreover, plants under water-deficit conditions exhibit the earliest protective process through the control of stoma closure by changing the turgor pressure in guard cells [15]. Several recent genetic studies have been performed with the overall goal of improving drought tolerance in crops by developing plants that are capable of reaching sufficient yields under drought-stress conditions [16]. In general, plants use three strategies, namely drought escape, drought avoidance, and drought tolerance, to mitigate the effects of drought stress [17]. Drought-response genes can themselves be grouped into two categories: genes involved in signal transduction and genes encoding functional components [18]. Plant drought responses are triggered by complex multicomponent signaling pathways. Several types of proteins that regulate drought tolerance have been identified in various plants species, such as dehydration-responsive element-binding proteins (DREBs), basic leucine-zipper (bZIP) transcription factors, and abscisic acid (ABA) receptors [17,18].

ABA is an important plant hormone that functions in plant responses to various stresses, including regulating stomata closure during drought stress [11]. Genes related to phytohormone biosynthesis, metabolism, and response play important roles in plant responses to drought stress [19]. ABA receptor genes, representing a large gene family in plants, play critical roles in plant development, signaling networks, and resistance to stress [20–23]. Several cytosolic ABA receptors have been functionally confirmed to confer drought tolerance. However, overexpressing ABA genes leads to ABA hypersensitivity, thereby inhibiting plant growth [21,24,25]. Regulating water use efficiency (WUE) in wheat by fine-tuning the expression of ABA receptor genes has provided a genetic approach for developing osmotic stress-tolerant crops without compromising total yields [26,27].

ABA receptors exist in monomeric and dimeric form. Dimeric ABA receptors require ABA to dissociate into monomers and form complexes with their downstream targets PP2CAs (Clade A Type-2C phosphatases), whereas monomeric ABA receptors may or may not require ABA to interact with PP2CAs [28–31]. *OsPYL/RCAR7* (pyrabactine resistance 1/PYR1-like/regulatory components of ABA receptor (PYR/PYL/RCAR7)), a monomeric ABA receptor in rice, has the lowest affinity with ABA and lowest ability to suppress OsPP2CA activity among all monomeric ABA receptors [32]. Overexpressing *OsPYL/RCAR7* did not lead to growth retardation, yield penalty, or major morphological changes in transgenic rice under normal growth conditions. However, under drought-stress conditions, young *OsPYL/RCAR7*-overexpressing rice seedlings showed a higher survival rate than the wild type did. Thus, perhaps the ABA receptor gene *OsPYL/RCAR7* could be used to enhance drought tolerance in crops without incurring any growth defects [32].

In this study, we overexpressed the ABA receptor gene *OsPYL/RCAR7* in maize, which resulted in enhanced drought tolerance for maize seedlings compared with nontransgenic plants. The transgenic maize plants did not show growth retardation or major morphological changes under normal growth conditions. Therefore, the *OsPYL/RCAR7* gene plays an important role in drought tolerance. This gene may serve as a useful tool for genetic manipulation to improve the agricultural properties of maize.

2. Material and Methods

2.1. Plant Materials and Genetic Transformation

Immature maize (*Zea mays*) Hi-II A embryos were used to produce transgenic plants [33]. The plants were grown in the greenhouse under a 16 h light/8 h dark cycle at 28/18 °C in Jeonju, Republic of Korea. A construct containing *OsPYL/RCAR7* under the control of the cauliflower mosaic virus (CaMV) 35S promoter reported by Bhatnagar et al. [32] was used to generate *OsPYL/RCAR7*-overexpressing (OX) transgenic maize (Figure S1a). The binary vector was used to stably transform immature maize Hi IIA embryos via a transformation mediated by *Agrobacterium* (*Agrobacterium tumefaciens*) (strain EHA101), as described previously [33,34]. T_0 plants were pollinated to produce seeds for subsequent

analysis. Male and female flower development can occur at different times in an individual T_0 plant. Therefore, maize B73 (nontransgenic donor plant) was used as pollen donor or recipient to cross with T_1 transgenic plants. F1 (B73 X *OsPYL/RCAR7*-OX) hybrid seedlings were used for the drought-stress assay.

2.2. Verification of Transgenic Maize

The accumulation of the herbicide resistance PAT protein (Phosphinothricin N-acetyltransferase) was analyzed using PAT-test strips (AgraStrip LL strip test, ROMER, Austria) to screen transgenic maize. In addition, to analyze the herbicide resistance of the transgenic lines, 0.3% (*v*/*v*) Basta was applied to part of each leaf after four weeks of growth by using a cotton swab or spraying it onto transgenic plants, and herbicide resistance was confirmed 19 days later [35].

In order to verify the presence of the transgenes in maize, PCR was performed using genomic DNA with a pair of *bar* gene primers (forward, 5′-TGCACCATCGTCAACCAC-3′; reverse, 5′-AGAAACCCACGTCATGCC-3′; 427 bp PCR fragment) and *OsPYL/RCAR7* gene primers (forward, 5′-ATGAACGGCGCTGGTGGTGCGGGAG-3′; reverse, 5′-TCAAGGATT GGCAAGGCGCTCCTC-3′; 621 bp PCR product). PCR was performed using 25 cycles of 30 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C, followed by a final extension at 72 °C for 5 min. The PCR fragments were confirmed by electrophoresis.

To identify the copy number of the T-DNA insertion, genomic DNA was subjected to PCR genotyping using TaqMan gene expression assays with *bar* gene primers (forward, 5′-GAGACAAGCACGGTCAACTTC-3′; reverse, 5′-CGAGGTCGTCCGTCCAC-3′; FAM- 5′-TCCTGCGGTTCCTGC-3′) and *ZmAdh1* gene primers (forward, 5′-GAATGTGTGTTGGGTT TGCAT-3′; reverse, 5′-TCCAGCAATCCTTGCACCTT-3′; VIC, 5′-TGCAGCCTAACCATGC GCAGGGTA-3′).

To confirm the T-DNA inserted position in transgenic maize, the extracted genomic DNA was digested with the restriction enzymes HincII (NEB, Ispwich, UK) and HaeIII (NEB, Ispwich, UK) and ligated to adaptors. PCR was performed using adaptor-specific primers. The PCR product was sequenced, and the portion of the sequence adjacent to the T-DNA insertion was confirmed by BLAST against MaizeGDB (https://www.maizegdb.org, accessed 12 August 2020) [36].

2.3. Postgermination Assay

Surface-sterilized transgenic F1 overexpressing *OsPYL/RCAR7* and wild-type seeds were plated on half-strength (1/2) MS medium and incubated for 3 days. Young seedlings were transferred to half-strength MS medium as the control and 1/2 MS medium containing 5 μM ABA (Duchefa, Haarlem, The Netherlands) or 200 mM mannitol (Duchefa, Haarlem, The Netherlands) for osmotic stress treatment. These plates were incubated for 7 days. In order to analyze the difference in plant growth, the root and shoot length were measured, and the average of the total measurements was calculated and graphed.

To analyze the expression of ABA-related genes at the postgermination growth stage, total RNA was extracted from 10-day-old seedlings grown on 1/2 MS medium treated with or without 5 or 20 μM ABA for 12 h. Total RNA was extracted from 200 mg of leaf from the transgenic plants using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). First-strand cDNA was synthesized from 5 μg of total RNA using MMLV reverse transcriptase (RNaseH-free; Toyobo, Osaka, Japan) according to the manufacturer's instructions. Gene expression was analyzed by using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) with primer sets specific to two ABA-related genes (*ZmABP2*, GRMZM2G033413, 5′-AGGACTTCGGCTCCATGAACATGGACG-3′, 5′-AGTGCTGAACGGATACGGCACCGGCG-3′; *ZmRAB16A*, GRMZM2G079440, 5′-GCAAGCATCATGGAGTACGGTCAGC-3′, and 5′-TCAGTGCTGTCCGGGCAGCTTCTCC-3′) [37–39]. Maize *ActinI* (GRMZM2G126010, J01238, NM_001155179.1) was used as a quantitative control to normalize transcript levels [13]. Each RT-qPCR assay was repeated three times. For statistical analysis, a Student's *t*-test was used.

2.4. Phenotypic Analysis under Drought Stress

Drought-tolerance analyses were performed according to previously described methods, with minor modifications [21,32]. Transgenic F1 overexpressing *OsPYL/RCAR7* and wild-type seeds were surface sterilized and germinated on MS medium for 3 days. Selected seedlings were transferred to soil and grown in an LED growth chamber (Eyela JP FLI-2000HT, Tokyo, Japan) for 28 days under a 16 h light/8 h dark cycle at 28/20 °C, 130 μmol/m^2/s, and 50% relative humidity. The plants were watered regularly during this period. The water supply was then suspended for 13 days when the plants had completely withered, and the watering resumed at this time. Data on the experiment in which the phenotypes were clearly visible were recorded for all samples. The survival rate was measured by counting the number of plants surviving in each pot. Three replicate pots were used for each transgenic plant line and wild-type combination.

2.5. Analysis of Water Loss and Stomatal Conductance

DroughtSpotter (Phenospex, Heerlen, The Netherlands) was used to measure the water loss of transgenic maize and wild-type plants in drought stress [40]. The plants were grown in a Phytotron (Korea Scientific Technique Industry, Suwon, Republic of Korea) under a 16 h light/8 h dark photoperiod, which is suitable for growing maize, at a daytime temperature of 28 °C with 450 μmol/m^2/s and a nighttime temperature of 20 °C with 50% relative humidity. A single 3-day-old seedling per GS120 pot (120 mm SQUAT Ketu plant pot) was grown in soil for 10 days prior to the drought-stress experiment. Before starting the measurement of total weight under drought stress, the total weight of each pot-containing plant, pot, and water-saturated soil was equalized to 630 g. The total weight limit of pots during irrigation was 630 g. Irrigation was carried out when the weight of the pot decreased by more than 5% of the original weight. An empty water-saturated soil pot without plants (empty pot) was used to measure the effect of the surrounding environment on the water evaporation from the soil. Plant water loss was calculated as the total water loss subtracted from the soil water loss at DSP 7 (7th day after the onset of the drought-stress phase [40].

Stomatal conductance was measured using a leaf porometer (SC-1, Decagon Devices, Pullman, WA) in DSP 7 and RWP 5 (5th day after the onset of the rewatering phase). It was measured from the abaxial side of each fully expanded leaf. Measurements were taken from 3 leaves per pot [41,42].

2.6. Statistical Analysis

One-way ANOVA test (Shapiro–Wilk normality statistic test) ($p < 0.01$) was performed for the measurement results of these experiments. In addition, the significance of differences in amounts between wild type and *OsPYL/RCAR7*-OX transgenic lines were tested using the Student's *t*-test ($p < 0.01$). It limited the range of values obtained from mean ± standard error (SE) with 95% confidence. In this study, Sigma plot 12.5 (Systat Software, GmbH, Germany) and the jamovi-1.8.1 (https://www.jamovi.org, accessed on 8 October 2022) program for Microsoft Windows was used for all statistical analyses.

3. Results

3.1. Generation of Transgenic Maize Overexpressing OsPYL/RCAR7

OsPYL/RCAR7 in rice is a functional monomeric ABA receptor with low ABA-mediated interaction affinity and low ability to inhibit the activity of OsPP2CAs. In a previous study, overexpressing *OsPYL/RCAR7* in rice improved resistance to drought stress without causing morphological changes such as growth retardation or reduced yield. Therefore, *OsPYL/RCAR7* may represent a unique ABA receptor gene that can improve drought tolerance without inhibiting plant growth in rice and perhaps other crops. In this study, we introduced the *OsPYL/RCAR7* gene into maize to generate plants with improved drought tolerance without growth defects. After transforming immature maize Hi-II A embryos with *Agrobacterium* containing a vector harboring *OsPYL/RCAR7*, we regenerated complete

plants from herbicide-resistant callus. The plants were then acclimatized to soil and grown in a greenhouse (Figure S1b–g). In immature embryo infected with *Agrobacterium*, the efficiency of callus formation on selection medium including bialaphos was 20.8% (data not shown). Twenty-three independent T_0 transformants were obtained using self-pollination (Figure S1h and Table S1).

We confirmed the expression of the introduced *Bar* gene in leaves of the regenerated T_0 transgenic plants using PAT-test strips (Figure S1i). We then selected transgenic plants harboring a single T-DNA insertion based on TaqMan analysis of genomic DNA extracted from the regenerated T_0 plants, and we harvested seeds (Figure S2 and Table S1). Sequencing of the region adjacent to T-DNA insertions in genomic DNA is required, as it provides important information about the stability of transformation [42,43]. To identify the position of the T-DNA insertion in the genomic DNA in three lines (#11–2, #12–26, and #13–2) and to confirm the insertion of a single copy of T-DNA in transgenic maize, we analyzed the nucleotide sequence adjacent to the T-DNA insertion in each line. We determined that the T-DNA insertions are located on chromosome 1 (between 190,570,614 bp and 190,570,049 bp), on chromosome 1 between 3,741,800 bp and 3,742,030 bp, or on chromosome 2 between 196,575,977 bp and 196,576,211 bp, respectively (Figure 1). These positions correspond to the first exon position, or 12.3 kb before the transcription start site of Zm00001d031453, Zm00001d027361, and Zm00001d006052, respectively. We selected two lines (#11–2 and #13–2) in which we predicted that the T-DNA did not directly induce the deletion or mutation of another gene or be inserted into an unknown gene. The seeds of T_0 plants were sown during the proper growing season for maize. After 4 weeks of growth, we tested the leaves of the T_1 plants with PAT-test strips and selected T_1 plants showing a positive reaction (Figure S3a). We then applied 0.3% of the herbicide Basta to the leaves of T_1 plants and selected individuals showing herbicide resistance 5 days after treatment (Figure S1j).

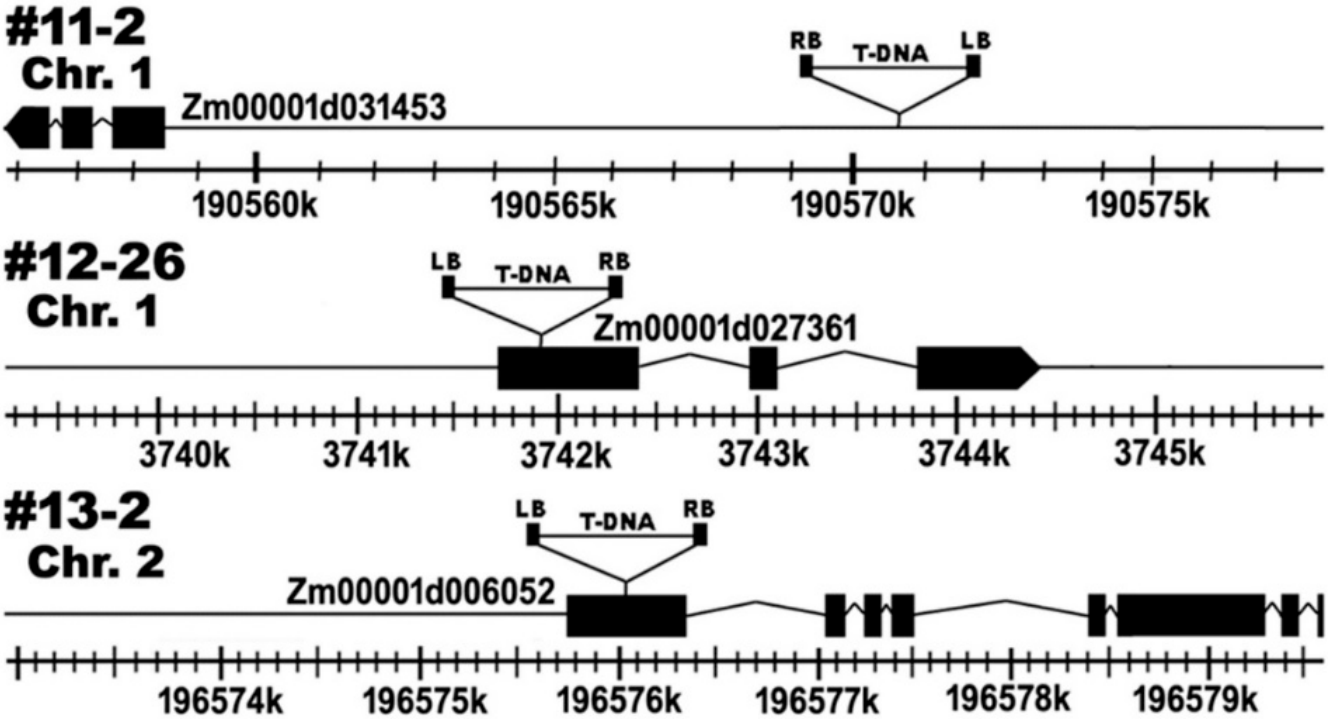

Figure 1. Positions of the T-DNA insertions in individual *OsPYL/RCAR7*-OX transgenic maize lines. Black boxes indicate exons. Numbers indicate chromosome positions. LB and RB indicate left and right borders of the T-DNA, respectively. #1101, #12–16, and #13–2 are the selected transgenic lines.

We crossed the progeny of the selected lines (#11–2 and #13–2) to plants from the maize inbred line B73 (Zm-*OsPYL/RCAR7*-OX ♀× B73 ♂and B73 ♀× Zm- *OsPYL/RCAR7*-OX ♂, named Type-A and Type-B, respectively) to produce the F_1 generation (Table S2). In normal

growth conditions, no significant differences in agricultural traits were found between the *OsPYL/RCAR7*-OX plants and the wild-type plants (data not shown). These results suggest that the overexpression of *OsPYL/RCAR7* shows no defect in maize growth under normal field conditions. We used PAT-test strips, genomic DNA PCR, RT-PCR, and a genotyping analysis of F_1 individuals to select individuals with a transgene ratio of 100% for drought-tolerance analysis (Figure S4 and Table 1). These lines were named according to their types: Type-A: 11A-5, 11A-8, 13A-5, 13A-9, and 13A-12; Type-B: 11B-5, 11B-8, 13B-5, 13B-9, and 13B-12.

Table 1. Summary of the genotypes of F_1 hybrid transgenic maize lines #11–2 and #13–2.

F_1—Type-A, B73 (♀) × T_1 (♂)					F_1—Type-B, T_1 (♀) × B73 (♂)				
Line (F_1)		Transgene Ratio (%)	Genotype	No. of Seeds	Line (F_1)		Transgene Ratio (%)	Genotype	No. of Seeds
#11–2-(named 11A-)	1	60	Ab, ab	191	#11–2-(named 11B-)	1	40	aB, ab	240
	2	0	ab	142		2	0	ab	237
	3	60	Ab, ab	187		3	50	aB, ab	262
	4	40	Ab, ab	158		4	40	aB, ab	267
	5	100	Ab	159		5	100	aB	239
	6	70	Ab, ab	177		6	40	aB, ab	365
	7	60	Ab, ab	142		7	50	aB, ab	270
	8	100	Ab	160		8	100	aB	238
#13–2-(named 13A-)	1	60	Ab, ab	202	#13–2-(named 13B-)	1	50	aB, ab	330
	2	0	ab	196		2	0	ab	289
	3	0	ab	191		3	0	ab	274
	4	40	Ab, ab	192		4	40	aB, ab	287
	5	100	Ab	199		5	100	aB	304
	6	50	Ab, ab	268		6	50	aB, ab	309
	7	40	Ab, ab	191		7	40	aB, ab	283
	8	70	Ab, ab	195		8	40	aB, ab	352
	9	100	Ab	197		9	100	aB	318
	10	60	Ab, ab	272		10	40	aB, ab	305
	11	50	Ab, ab	442		11	40	aB, ab	354
	12	100	Ab	239		12	100	aB	345

Ab and aB, heterozygous for the single T-DNA insertion; ab, wild type.

3.2. Phenotypes of OsPYL/RCAR7-OX Transgenic Maize

We performed various experiments to determine whether overexpressing *OsPYL/RCAR7* was associated with enhanced drought tolerance in maize plants grown in soil in pots. The growth of transgenic seedlings was similar to that of the wild type under normal conditions. All plants showed leaf wilting following 10 days of water withholding and exhibited severe wilting after 3 more days of drought treatment (Figure 2a). However, the wild-type seedlings were dehydrated and some died after this treatment, whereas *OsPYL/RCAR7*-OX seedlings were relatively green and less wilted than the wild type. After rewatering, the *OsPYL/RCAR7*-OX plants fully recovered, whereas all wild-type plants died. *OsPYL/RCAR7*-OX transgenic maize showed higher survival rates than the wild type, with an average survival rate of 50–75% (Figure 2b). Among the 10 transgenic lines, we chose six lines (Type-A: 11A-5, 13A-5, and 13A-9; Type-B: 11B-5, 13B-5, and 13B-9) for further study.

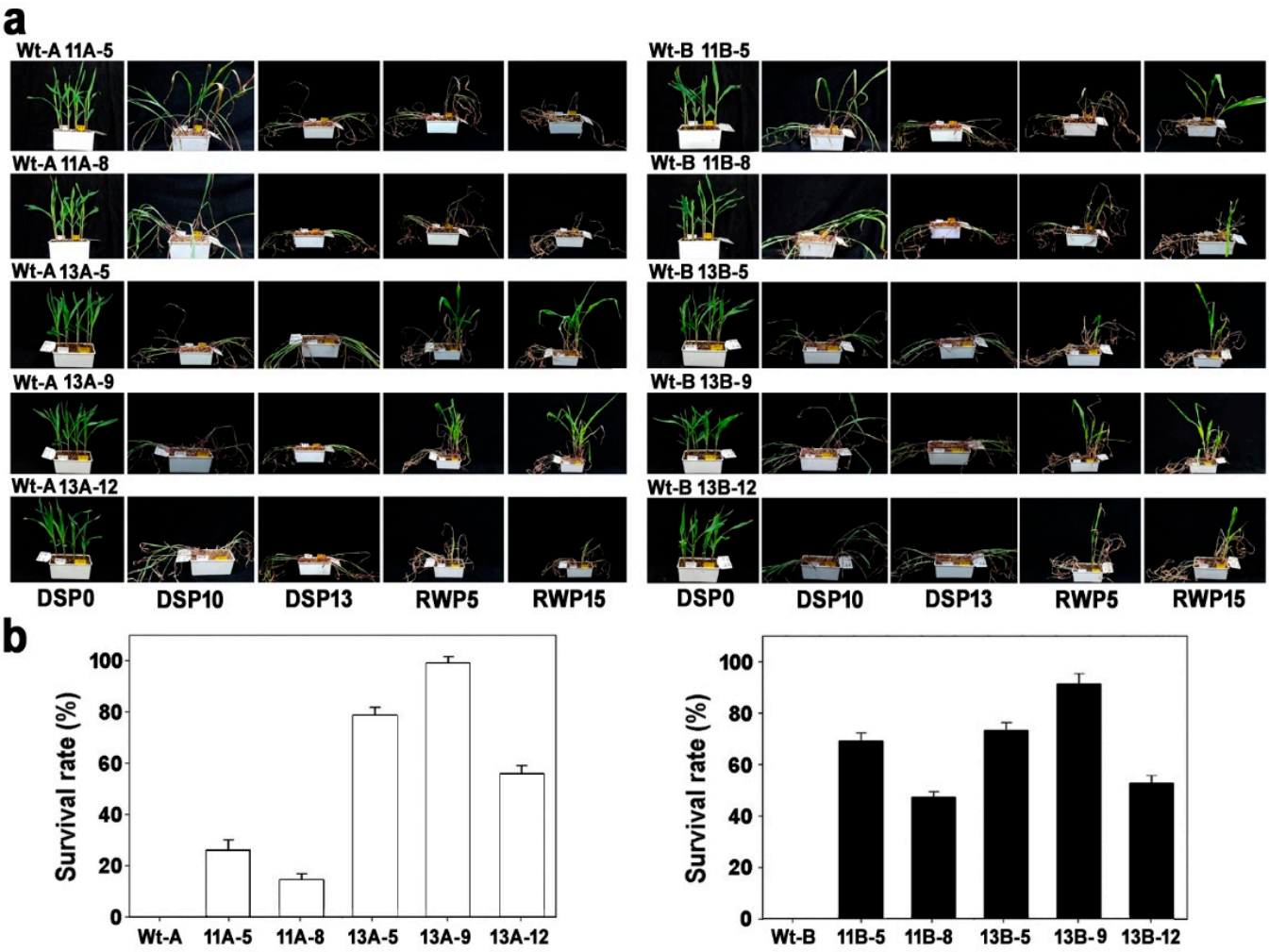

Figure 2. Tolerance of *OsPYL/RCAR7*-OX maize seedlings to drought stress. (**a**) Seedlings grown of transgenic maize under drought-stress conditions. (**b**) Survival rate after drought stress. Survival rate was calculated by counting the surviving seedlings in each pot. Bars represent the means ± SE of three replicates. DSP, drought-stress phase; RWP, rewatering phase; Wt, untransformed wild-type plants; and 11A-5 to 13B-12, selected transgenic lines.

In a previous study, rice plants overexpressing the ABA receptor gene *OsPYL/RCAR7* showed no growth retardation, had a high survival rate under drought stress, and showed improved drought resistance because of delayed water loss, exhibiting increased tolerance to ABA and osmotic stress. Therefore, we performed post-germination assays to determine whether *OsPYL/RCAR7*-OX transgenic maize would also show enhanced ABA and osmotic stress tolerance using 10-day-old seedlings (Figure 3a). The responses of the *OsPYL/RCAR7*-OX seedlings under normal or stress conditions were not significantly different from those of the wild type. No significant difference between *OsPYL/RCAR7*-OX transgenic maize and wild type was observed in the length of shoots and roots (Figure 3b). These results suggest that, like *OsPYL/RCAR7*-OX lines in rice, maize plants overexpressing *OsPYL/RCAR7* showed no defect in ABA and osmotic stress.

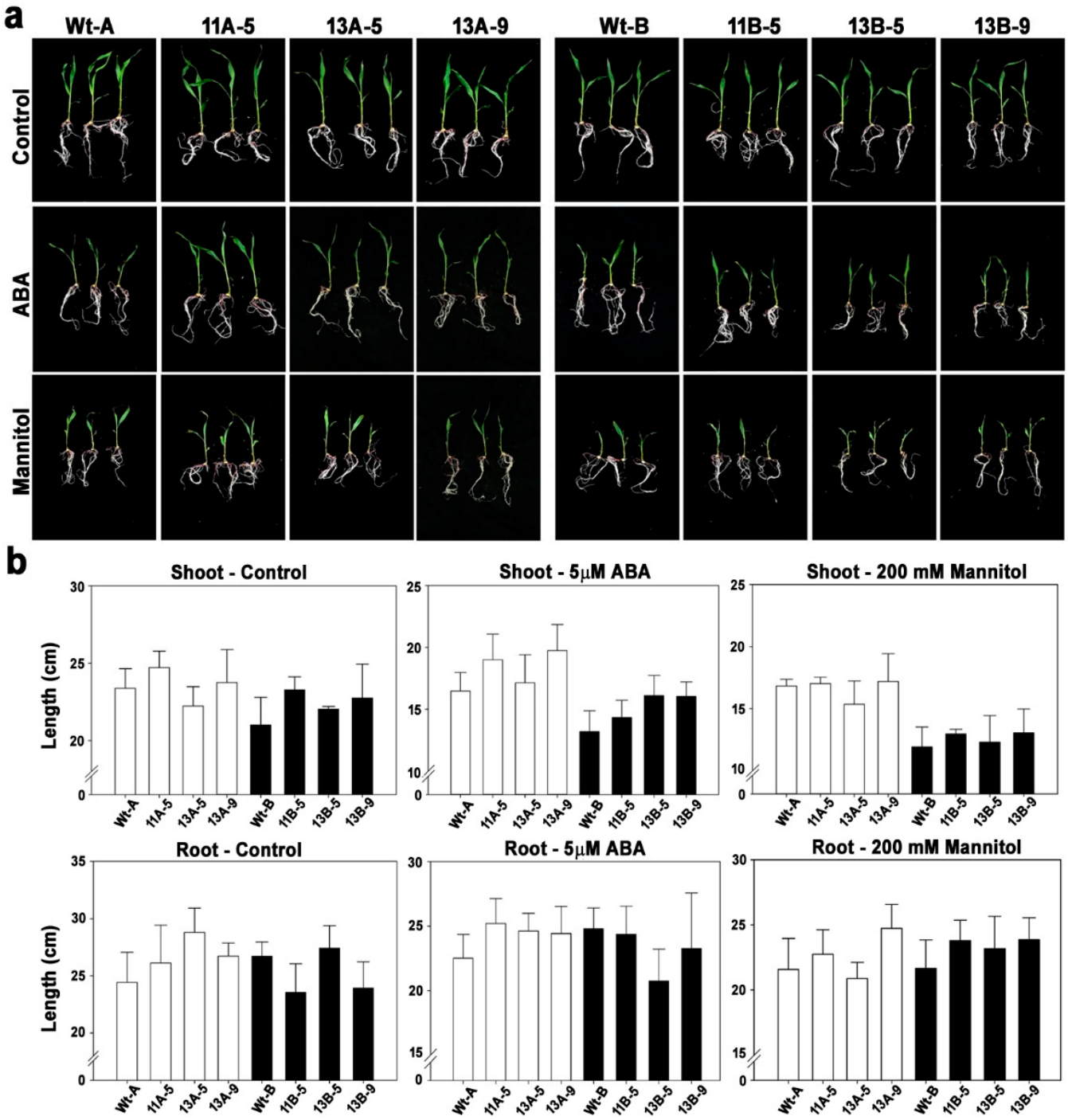

Figure 3. Post-germination assay of *OsPYL/RCAR7*-OX transgenic maize lines under abscisic acid (ABA) treatment and osmotic stress conditions. (**a**) *OsPYL/RCAR7*-OX transgenic and wild-type seedlings grown on half-strength MS medium containing 5 μM ABA or 200 mM mannitol. (**b**) Root and shoot lengths of seedlings from the postgermination assay. Bars represent the means ± SE of three replicates. Wt, untransformed wild-type seedlings; and 11A-5 to 13B-12, selected transgenic lines.

3.3. Analysis of Water Loss and Stomatal Conductance

Because *OsPYL/RCAR7*-OX transgenic maize displayed enhanced drought tolerance, we reasoned that these plants might possess more positive traits in terms of WUE than the wild type. We therefore analyzed the water consumption of *OsPYL/RCAR7*-OX transgenic maize by using the DroughtSpotter (PHENOSPEX) platform and a leaf porometer in plants grown in a Phytotron in which the temperature, relative humidity, and LED light levels

were precisely controlled [44]. The Phytotron conditions for this experiment are shown in Figure S5.

Under control conditions, pots containing 13-day-old seedlings and water-saturated soil weighed 630 g. When the total weight decreased by more than 5% of the original weight, the water was replenished [40]. After watering had been terminated, the pots were weighed in 1 min intervals every day. Empty pots, with no seedling and containing only water-saturated soil, were weighed at the same time as pots containing wild-type and transgenic seedlings. Under drought-stress conditions, the total weight was higher for *OsPYL/RCAR7*-OX transgenic maize than for the wild type (Figure 4a). We observed the greatest difference at DSP 7: the total weight was ~300 g for the wild type and ~366 g for the transgenic plants, a difference of approximately 66 g. The total weight of empty pots was ~472 g at DSP 7. According to these results, the average plant water loss was about 172 g in the wild-type plants and about 106 g in the transgenic plants. Therefore, the water loss of whole plants was approximately 1.6 times higher in the wild type than in *OsPYL/RCAR7*-OX seedlings (Figure S6). Therefore, under drought stress, wild-type seedlings consumed more water than transgenic plants did.

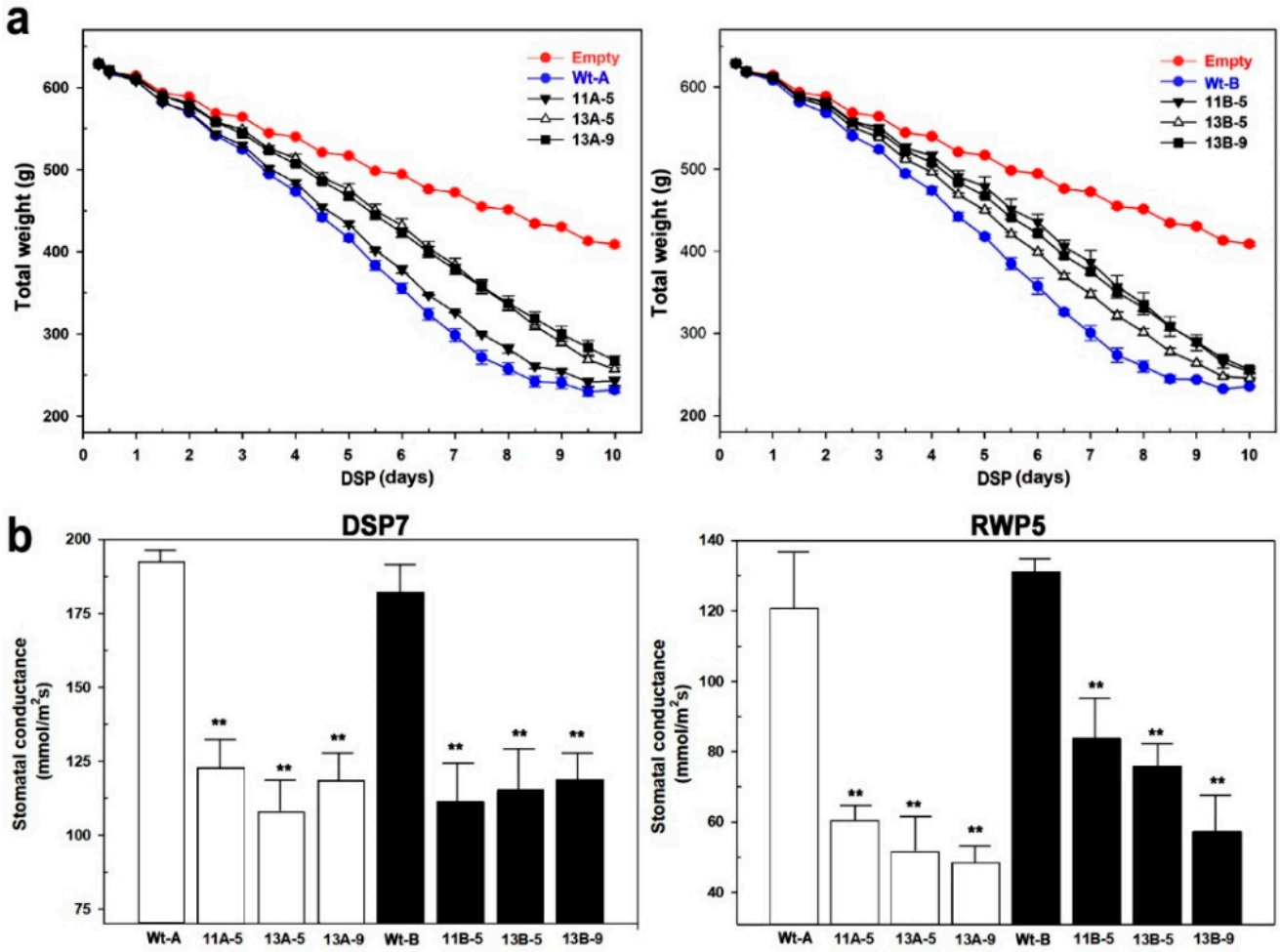

Figure 4. Water loss and stomatal conductance of *OsPYL/RCAR7*-OX transgenic maize lines under drought-stress conditions. (**a**) Changes in the total weight of pots with seedlings (pots containing wild-type or *OsPYL/RCAR7*-OX seedlings) from DSP0 to DSP10. (**b**) Stomatal conductance of wild-type and *OsPYL/RCAR7*-OX seedlings at DSP 7 and RWP 5. Bars represent the means ± SE of three replicates. Asterisks represent significant differences from the wild type (**, *p*-value < 0.01). DSP, drought-stress phase; RWP, rewatering phase; Wt, untransformed wild-type plants; and 11A-5 to 13B-12, selected transgenic lines.

Given the above results, we measured the stomatal conductance at DSP 7. The stomatal conductance was approximately 1.6 times higher in the wild-type seedlings than the transgenic seedlings (Figure 4b). During this phase, wild-type plants were severely drought stressed and failed to exhibit normal stomatal opening and closure, suggesting that stomatal conductance was higher in these plants than the transgenic plants. On the 3rd day after rewatering, the transgenic seedlings recovered more quickly than the wild type (Figure S6). In addition, stomatal conductance was lower in the transgenic seedlings than in the wild type (Figure 4b). These results suggest that *OsPYL/RCAR7*-OX transgenic maize seedlings are resistant to drought thanks to the effects of this gene on stomatal opening and closure.

3.4. Expression Patterns of ABA-Related Genes in Transgenic Maize Plants

A previous study has shown that ABA-dependently-induced genes expressed in the *OsPYL/RCAR7*-OX transgenic rice of responded more sensitively to ABA than the wild type did when ABA was treated for 12 h [32]. To gain insights into the molecular basis of the drought tolerance associated with *OsPYL/RCAR7* overexpression and to examine the role of this gene in drought-stress signaling, we compared the expression profiles of several well-characterized ABA-inducible genes, such as *ABP2* (*ABRE binding protein 2*) and *RAB16A* (responsive to ABA 16A) [37,38] in 10-day-old wild-type and *OsPYL/RCAR7*-OX transgenic maize grown with or without ABA treatment for 12 h. As shown in Figure 5, overexpressing *OsPYL/RCAR7* resulted in the accumulation of *ABP2* and *RAB16A* transcripts under normal conditions, suggesting that *OsPYL/RCAR7* might be a positive upstream regulator of these genes in the drought-stress-signaling pathway. After 12 h of ABA treatment, *RAB16A* was expressed at higher levels in *OsPYL/RCAR7*-OX seedlings than in the wild type. However, *ABP2* expression was strongly reduced in *OsPYL/RCAR7*-OX transgenic seedlings when the ABA concentration increased. These results indicate that these two genes respond more strongly to ABA in the *OsPYL/RCAR7*-OX lines than the wild type does.

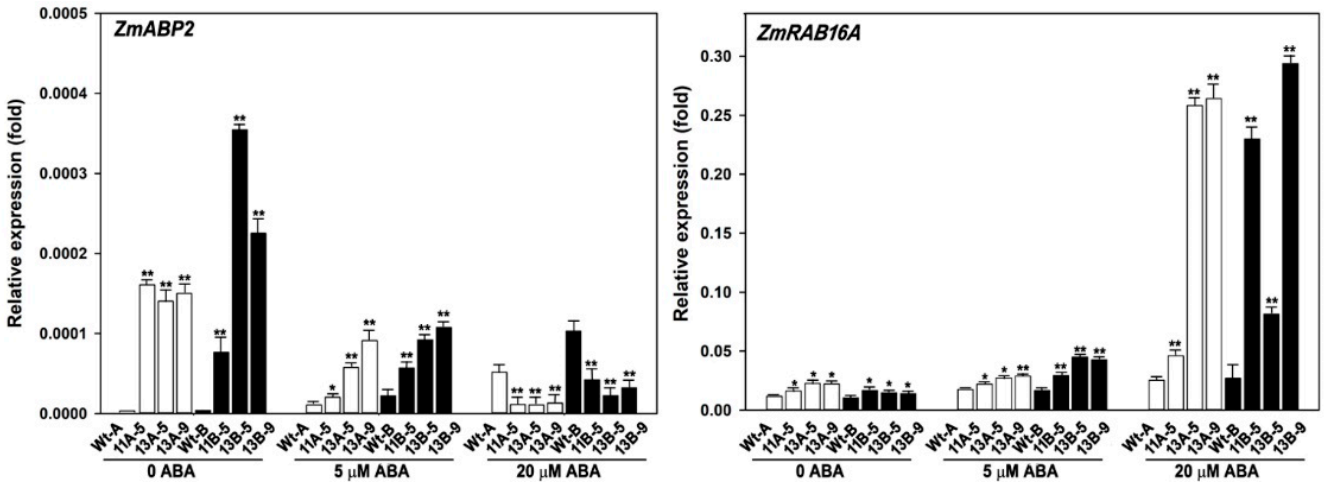

Figure 5. Expression patterns of ABA-inducible genes in *OsPYL/RCAR7*-OX transgenic maize lines. Each transcript was detected in seedlings treated with the indicated concentration of ABA for 12 h. Bars represent the means ± SE of three replicates. Asterisks represent significant differences from the untransformed wild type (*, 0.01 < *p*-value < 0.05; **, *p*-value < 0.01). Wt, untransformed wild type; 11A-5 to 13B-12, selected transgenic lines.

4. Discussion

Drought is currently one of the most harmful abiotic stresses affecting agriculture. Maize is a multipurpose crop grown worldwide that on a yearly basis suffers yield losses due to drought [45]. Several drought-tolerance-enhancing genes have been reported in maize [13,35]; the effects of these genes on agricultural traits have not been systematically studied, making it difficult to use these genes in practice. Therefore, it is essential to identify genes that confer drought tolerance while maintaining excellent agricultural traits in maize.

ABA, an important phytohormone involved in plants' drought tolerance, functions via a signal transduction cascade involving receptors (OsPYL/RCARs), phosphatases (OsPP2Cs), and kinases (sucrose nonfermenting-related kinase 2 [SnRK2]) [28–31]. ABA signaling is a key pathway that regulates the responses of crops to environmental stresses such as drought. The formation of complexes via the interactions of ABA receptors with phosphatases is essential for regulating the initiation of ABA signaling [29,46]. Most transgenic plants overexpressing ABA receptor genes show enhanced drought tolerance but poor growth or decreased yields because of negative effects on agricultural traits [21,24,45]. However, transgenic rice overexpressing *OsPYL/RCAR7*, encoding an ABA receptor with low signaling activity in rice, showed enhanced drought tolerance but similar agricultural traits compared with those of the wild type [32].

The aim of the current study was to improve the drought tolerance of maize by transgenically expressing the *OsPYL/RCAR7* gene into this crop. We generated transgenic maize plants overexpressing *OsPYL/RCAR7* through *Agrobacterium*-mediated transformation and examined whether the target gene was inserted into an intergenic region or an unknown gene in the genome as a single copy gene in T_0 plants. Among the transgenic plants, we selected two for further study. Unfortunately, T_0 Hi-II A transgenic maize had poor agricultural traits and showed very low seed formation and yield after self-crossing (Figure S3b and Table S1). To solve this problem, following the 'Greenhouse care for transgenic maize plants' protocol (http://agron-www.agron.iastate.edu/ptf/protocol/Greenhouse%20Protocol.pdf, accessed on 8 October 2022), we crossed the transgenic Hi-II A line to the maize inbred line B73 and used the resulting F1 hybrid seeds to generate additional experimental materials (Figure S3b, Tables 1 and S2).

Overexpressing *OsPYL/RCAR7* resulted in enhanced tolerance to drought treatment (Figures 2 and S7). In addition, the *OsPYL/RCAR7*-OX plants showed no seedling growth retardation under normal conditions (Figure 3). The phenotypes of these *OsPYL/RCAR7*-OX maize plants can be explained by the differential expression of ABA-responsive/stress-tolerance-related genes compared with the wild type. In a previous study, overexpressing the ABA-related gene *ABP2* (encoding a bZIP transcription factor) in *Arabidopsis thaliana* led to normal growth but enhanced drought tolerance by modulating the ABA-mediated balance of reactive oxygen species and stomatal aperture, thereby reducing cellular damage [35]. *RAB16A*, which functions downstream of ABA signaling, is also involved in plant responses to drought stress [38,47,48].

Here, overexpressing *OsPYL/RCAR7* increased the transcript levels of *ABP2* and *RAB16A* compared with the wild type under normal growth conditions, but not under ABA treatment (Figure 5). Under ABA treatment, *RAB16A* was upregulated by *OsPYL/RCAR7* overexpression, whereas *ABP2* was strongly downregulated (Figure 5). These results suggest that introducing the *OsPYL/RCAR7* gene into maize enhanced the drought tolerance of seedlings by directly or indirectly regulating the expression of the drought-tolerance-related genes *ABP2* and *RAB16A*. The different expression patterns of *ABP2* and *RAB16A* in response to ABA might be important for balancing plant growth/productivity and stress tolerance [32]. Nonetheless, expressing *OsPYL/RCAR7* in maize led to the upregulation of stress-responsive genes under normal conditions; the stress-responsive genes *ABP2* and *RAB16A* were highly induced in these plants, suggesting that *OsPYL/RCAR7* enhances drought-stress tolerance in maize seedlings by modulating the ABA-mediated stomatal aperture.

The water content of plants is a major physiological indicator that reflects their ability to withstand stressful environments [49]. We quantified the physiological responses of plants to drought by measuring water loss and stomatal conductance using the DroughtSpotter platform and a leaf porometer, which helped us characterize the actual drought tolerance of *OsPYL/RCAR7*-OX transgenic maize seedlings. As shown in Figure 4a, water loss was significantly greater in the wild type than in drought-tolerant *OsPYL/RCAR7*-OX transgenic maize, suggesting that the transgenic plants required less water than the wild type (Figure S6). These results indicate that overexpressing *OsPYL/RCAR7* in maize

seedlings improved drought-stress tolerance by enhancing the water-retention capacity of the plant.

Drought stress occurs when plants have a high rate of sustained water loss via transpiration [50]. Stomata are epidermal valves that play central roles in regulating drought tolerance and plant water loss to control the utilization of water [51]. Under drought stress, plants control stomatal closure as a defense response to minimize water loss [52]. The *OsPYL/RCAR7*-OX maize plants showed reduced stomatal conductance compared with the wild type (Figure 4b). Similarly, ZmABP2 regulates stomatal aperture to increase drought-stress tolerance in plants [38]; the constitutive expression of *ZmABP2* in Arabidopsis led to enhanced drought tolerance by affecting ABA-mediated stomatal opening and closure. Finally, under normal conditions, the *OsPYL/RCAR7*-OX transgenic plant growth did not significantly differ from that of the wild type. These findings suggest that *OsPYL/RCAR7* plays an important role in conserving water in plants by regulating the stomatal aperture. Although our data do not clarify whether the *OsPYL/RCAR7*-OX influenced mature maize plants phenotypes, they do suggest that the drought-tolerance phenotypes in maize seedlings were enhanced by the *OsPYL/RCAR7*. However, the detailed function of *OsPYL/RCAR7* in drought tolerance during maize vegetative and reproductive stage remains to be elucidated, so further experiments are required to define their complete physiological functions in response to drought tolerance.

In this study, we demonstrated that overexpressing *OsPYL/RCAR7* improved the drought tolerance of maize seedlings without obvious negative effects on plant growth and development. Consequently, when the soil water became limiting, *OsPYL/RCAR7*-OX plants were better able to avoid dehydration than the wild-type plants were.

5. Conclusions

In conclusion, overexpressing *OsPYL/RCAR7* improved drought tolerance in maize by reducing water loss and stomatal conductance without growth defects, as in rice. We demonstrated that this gene regulates the expression of ABA-related genes controlling the plant's drought response and sensitivity to ABA. Taken together, our data indicated that *OsPYL/RCAR7* enhanced drought tolerance in maize, raising the possibility that this gene may serve as a useful genetic resource for crop improvement to help plants adapt to local environments and the changing climate.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/agriculture12122140/s1, Figure S1. Plant regeneration from immature Hi IIA embryo cultures transformed with *Agrobacterium* carrying a construct for the expression of *OsPYL/RCAR7*. Figure S2. Analysis of copy number variation in T_0 *OsPYL/RCAR7*-OX transgenic maize using TaqMan assays. Figure S3. Hybrid seed production in T_1 transgenic maize. Figure S4. *OsPYL/RCAR7* gene expression in F_1 transgenic maize. Figure S5. Examining water loss from whole plants using the DroughtSpotter platform in an environmentally controlled Phytotron. Figure S6. Water loss rates of *OsPYL/RCAR7*-OX transgenic maize lines at DSP7. Figure S7. Tolerance of *OsPYL/RCAR7*-OX transgenic maize lines to drought stress in an environmentally controlled Phytotron. Table S1. Information about harvested T_0 *OsPYL/RCAR7*-OX transgenic maize. Table S2. The first generation (F_1) of a cross between *OsPYL/RCAR7*-OX transgenic T_1 maize and B73 plants.

Author Contributions: S.R.P. conceived and designed the research; G.S. operated the leaf porometer system; J.K.H. and H.J.J. performed experiments; J.K.H., Y.-H.L., E.J.S., G.S.L. and B.-G.K. collected and analyzed the data; and J.K.H. and S.R.P. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This work was carried out with the support of Research Program for Agricultural Biotechnology (PJ01597602), National Institute of Agricultural Sciences, Rural Development Administration, Republic of Korea. This study was supported in 2022 by the RDA Fellowship Program (Project No. PJ01570602) of National Institute of Agricultural Sciences, Rural Development Administration, Republic of Korea.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Huang, C.L. Maize and grace: Africa's encounter with a New World crop, 1500–2000—By James, C.; McCann. *Dev. Econ.* **2007**, *45*, 242–245.
2. Mueller, N.D.; Gerber, J.S.; Johnston, M.; Ray, D.K.; Ramankutty, N.; Foley, J.A. Closing yield gaps through nutrient and water management. *Nature* **2012**, *490*, 254–257. [CrossRef]
3. Edgerton, M.D. Increasing crop productivity to meet global needs for feed, food, and fuel. *Plant Physiol.* **2009**, *149*, 7–13. [CrossRef]
4. Khatoon, T.; Hussain, K.; Majeed, A.; Nawaz, K.; Nisar, M.F. Morphological variations in maize (*Zea mays* L.) under different levels of NaCl at germination stage. *World Appl. Sci. J.* **2010**, *8*, 1294–1297.
5. Nepolean, T.; Kaul, J.; Mukri, G.; Mittal, S. Genomics enabled next-generation breeding approaches for developing system-specific drought tolerant hybrids in maize. *Front. Plant Sci.* **2018**, *9*, 361. [CrossRef]
6. Farooq, M.; Hussain, M.; Wakeel, A.; Siddique, K.H. Salt stress in maize: Effects, resistance mechanisms, and management. A review. *Agron. Sustain. Dev.* **2015**, *35*, 461–481. [CrossRef]
7. Sun, Y.; Mu, C.; Zheng, H.; Lu, S.; Zhang, H.; Zhang, X.; Liu, X. Exogenous Pi supplementation improved the salt tolerance of maize (*Zea mays* L.) by promoting Na^+ exclusion. *Sci. Rep.* **2018**, *8*, 16203. [CrossRef]
8. Adhikari, B.; Sa, K.J.; Lee, K. Drought tolerance screening of maize inbred lines at an early growth stage. *Plant Breed. Biotechnol.* **2019**, *7*, 326–339. [CrossRef]
9. Li, Z.; Liu, C.; Zhang, Y.; Wang, B.; Ran, Q.; Zhang, J. The bHLH family member ZmPTF1 regulates drought tolerance in maize by promoting root development and abscisic acid synthesis. *J. Exp. Bot.* **2019**, *70*, 5471–5486. [CrossRef]
10. Mehta, R.H.; Ponnuchamy, M.; Kumar, J.; Reddy, N.R. Exploring drought stress-regulated genes in senna (*Cassia angustifolia* Vahl.): A transcriptomic approach. *Funct. Integr. Genom.* **2017**, *17*, 1–25. [CrossRef]
11. Bray, E.A. Plant responses to water deficit. *Trends Plant Sci.* **1987**, *2*, 48–54. [CrossRef]
12. Dai, A. Increasing drought under global warming in observations and models. *Nat. Clim. Chang.* **2013**, *3*, 52–58. [CrossRef]
13. Chaves, M.M.; Maroco, J.P.; Pereira, J.S. Understanding plant responses to drought-from genes to the whole plant. *Funct. Plant Biol.* **2003**, *30*, 239–264. [CrossRef]
14. Zhang, J.; Jiang, H.; Song, X.; Jin, J.; Zhang, X. The responses of plant leaf CO_2/H_2O exchange and water use efficiency to drought: A meta-analysis. *Sustainability* **2018**, *10*, 551. [CrossRef]
15. Zenda, T.; Liu, S.; Wang, X.; Liu, G.; Jin, H.; Dong, A.; Yang, Y.; Duan, H. Key maize drought-responsive genes and pathways revealed by comparative transcriptome and physiological analysis of contrasting inbred lines. *Int. J. Mol. Sci.* **2019**, *20*, 1268. [CrossRef]
16. Jewell, M.C.; Campbell, B.C.; Godwin, I.D. Transgenic plants for abiotic stress resistance. In *Transgenic Crop Plants*; Kole, C., Michler, C.H., Abbott, A.G., Hall, T.C., Eds.; Springer: Berlin/Heidelberg, Germany, 2010; pp. 67–132.
17. Nakashima, K.; Yamaguchi-Shinozaki, K.; Shinozaki, K. The transcriptional regulatory network in the drought response and its crosstalk in abiotic stress responses including drought, cold, and heat. *Front. Plant Sci.* **2014**, *5*, 170. [CrossRef]
18. Joshi, R.; Wani, S.H.; Singh, B.; Bohra, A.; Dar, Z.A.; Lone, A.A.; Pareek, A.; Singla-Pareek, S.L. Transcription factors and plants response to drought stress: Current understanding and future directions. *Front. Plant Sci.* **2016**, *7*, 1029. [CrossRef]
19. Jones, A.M. A new look at stress: Abscisic acid patterns and dynamics at high-resolution. *New Phytol.* **2015**, *210*, 38–44. [CrossRef]
20. Kim, H.; Hwang, H.; Hong, J.W.; Lee, Y.N.; Ahn, I.P.; Yoon, I.S.; Yoo, S.D.; Lee, S.; Lee, S.C.; Kim, B.G. A rice orthologue of the ABA receptor, OsPYL/RCAR5, is a positive regulator of the ABA signal transduction pathway in seed germination and early seedling growth. *J. Exp. Bot.* **2012**, *63*, 1013–1024. [CrossRef]
21. Kim, H.; Lee, K.; Hwang, H.; Bhatnagar, N.; Kim, D.Y.; Yoon, I.S.; Byun, M.O.; Kim, S.T.; Jung, K.H.; Kim, B.G. Overexpression of PYL5 in rice enhances drought tolerance, inhibits growth, and modulates gene expression. *J. Exp. Bot.* **2014**, *65*, 453–456. [CrossRef]
22. He, Y.; Hao, Q.; Li, W.; Yan, C.; Yan, N.; Yin, P. Identification and characterization of ABA receptors in *Oryza sativa*. *PLoS ONE* **2014**, *9*, e95246. [CrossRef] [PubMed]
23. Yang, Z.; Liu, J.; Tischer, S.V.; Christmann, A.; Windisch, W.; Schnyder, H.; Grill, E. Leveraging abscisic acid receptors for efficient water use in Arabidopsis. *Proc. Natl. Acad. Sci. USA* **2016**, *113*, 6791–6796. [CrossRef] [PubMed]
24. Miao, C.; Xiao, L.; Hua, K.; Zou, C.; Zhao, Y.; Bressan, R.A.; Zhu, J.K. Mutations in a subfamily of abscisic acid receptor genes promote rice growth and productivity. *Proc. Natl. Acad. Sci. USA* **2018**, *115*, 6058–6063. [CrossRef] [PubMed]
25. Yu, J.; Yang, L.; Liu, X.; Tang, R.; Wang, Y.; Ge, H.; Wu, M.; Zhang, J.; Zhao, F.; Luan, S.; et al. Overexpression of poplar pyrabactin resistance-like abscisic acid receptors promotes abscisic acid sensitivity and drought resistance in transgenic Arabidopsis. *PLoS ONE* **2016**, *11*, e0168040. [CrossRef]
26. Mega, R.; Tsujimoto, H.; Okamoto, M. Genetic manipulation of abscisic acid receptors enables modulation of water use efficiency. *Plant Signal. Behav.* **2019**, *14*, e1642039. [CrossRef]

27. Mega, R.; Abe, F.; Kim, J.S.; Tsuboi, Y.; Tanaka, K.; Kobayashi, H.; Sakata, Y.; Hanada, K.; Tsujimoto, H.; Kikuchi, J.; et al. Tuning water-use efficiency and drought tolerance in wheat using abscisic acid receptors. *Nat. Plants* **2019**, *5*, 153–159. [CrossRef]
28. Santiago, J.; Rodrigues, A.; Saez, A.; Rubio, S.; Antoni, R.; Dupeux, F.; Park, S.Y.; Márquez, J.A.; Cutler, S.R.; Rodriguez, P.L. Modulation of drought resistance by the abscisic acid receptor PYL5 through inhibition of clade A PP2Cs. *Plant J.* **2009**, *60*, 575–588. [CrossRef]
29. Park, S.Y.; Peterson, F.C.; Mosquna, A.; Yao, J.; Volkman, B.F.; Cutler, S.R. Agrochemical control of plant water use using engineered abscisic acid receptors. *Nature* **2015**, *520*, 545–548. [CrossRef]
30. Dorosh, L.; Kharenko, O.A.; Rajagopalan, N.; Loewen, M.C.; Stepanova, M. Molecular mechanisms in the activation of abscisic acid receptor PYR1. *PLoS Comput. Biol.* **2013**, *9*, e1003114. [CrossRef]
31. Nakagawa, M.; Kagiyama, M.; Shibata, N.; Hirano, Y.; Hakoshima, T. Mechanism of high-affinity abscisic acid binding to PYL9/RCAR1. *Genes Cells* **2014**, *19*, 386–404. [CrossRef]
32. Bhatnagar, V.; Kim, R.; Han, S.; Song, J.; Lee, G.S.; Lee, S.; Min, M.K.; Kim, B.-G. Ectopic Expression of OsPYL/RCAR7, an ABA receptor having low signaling activity, improves drought tolerance without growth defects in rice. *Int. J. Mol. Sci.* **2020**, *21*, 4163. [CrossRef] [PubMed]
33. Hong, J.K.; Lee, G.-S.; Park, K.J.; Kim, J.-K.; Jang, H.J.; Suh, E.J.; Kim, K.-H.; Lee, Y.H. Production of transgenic maize plants with herbicide resistance through *Agrobacterium*-mediated transformation. *Korean J. Breed. Sci.* **2019**, *51*, 290–297. [CrossRef]
34. Lee, H.; Zhang, Z.J. Maize (*Zea mays*) Hi-II transformation via *Agrobacterium*-mediated T-DNA transfer. *Curr. Protoc. Plant Biol.* **2016**, *1*, 121–137. [CrossRef]
35. Cho, M.J.; Banh, J.; Yu, M.; Kwan, J.; Jones, T.J. Improvement of *Agrobacterium*-mediated transformation frequency in multiple modern elite commercial maize (*Zea mays* L.) inbreds by media modifications. *Plant Cell Tissue Organ Cult.* **2015**, *121*, 519–529. [CrossRef]
36. Hong, J.K.; Park, S.-R.; Suh, E.J.; Park, J.; Lee, H.-H. Effects of overexpression of *Brassica rapa SHORT VEGETATIVE PHASE* gene on flowering time. *Korean J. Breed. Sci.* **2020**, *52*, 244–251. [CrossRef]
37. Zong, N.; Li, X.-J.; Wang, L.; Wang, Y.; Wen, H.-T.; Li, L.; Zhang, X.; Fan, Y.-L.; Zhao, J. Maize *ABP2* enhances tolerance to drought and salt stress in transgenic *Arabidopsis*. *J. Integr. Agric.* **2018**, *17*, 2379–2393. [CrossRef]
38. Gao, Y.; Wu, M.; Zhang, M.; Jiang, W.; Ren, X.; Liang, E.; Zhang, D.; Zhang, C.; Xiao, N.; Li, Y.; et al. A maize phytochrome-interacting factors protein ZmPIF1 enhances drought tolerance by inducing stomatal closure and improves grain yield in *Oryza sativa*. *Plant Biotechnol. J.* **2018**, *16*, 1375–1387. [CrossRef] [PubMed]
39. Hong, J.K.; Suh, E.J.; Park, S.R.; Park, J.; Lee, Y.-H. Multiplex CRISPR/Cas9 mutagenesis of *BrVRN1* delays flowering time in Chinese cabbage (*Brassica rapa* L. ssp. *Pekinensis*). *Agriculture* **2021**, *11*, 1286. [CrossRef]
40. Kim, S.L.; Kim, N.; Lee, H.; Lee, E.; Cheon, K.-S.; Kim, M.; Baek, J.; Choi, I.; Ji, H.; Yoon, I.S.; et al. High-throughput phenotyping platform for analyzing drought tolerance in rice. *Planta* **2020**, *252*, 38. [CrossRef]
41. Ravia, S.; Young, T.; Macinnis-Ngc, C.; Nyugena, T.V.; Duxburya, M.; Alfaroa, A.C.; Leuzingera, S. Untargeted metabolomics in halophytes: The role of different metabolites in New Zealand mangroves under multi-factorial abiotic stress conditions. *Environ. Exp. Bot.* **2020**, *173*, 103993. [CrossRef]
42. Carvalhoa, H.D.R.; Heilmana, J.L.; McInnesa, K.J.; Rooneya, W.L.; Lewis, K.L. Epicuticular wax and its effect on canopy temperature and water use of Sorghum. *Agric. For. Meteorol.* **2020**, *284*, 107893. [CrossRef]
43. Spalinskas, R.; Bulcke, M.; Eede, G.; Milcamps, A. LT-RADE: An efficient user-friendly genome walking method applied to the molecular characterization of the insertion site of genetically modified maize MON810 and rice LLRICE62. *Food Anal. Methods* **2013**, *6*, 705–713. [CrossRef]
44. Park, S.-I.; Kwon, H.J.; Cho, M.H.; Song, J.S.; Kim, B.-G.; Baek, J.H.; Kim, S.L.; Ji, H.S.; Kwon, T.-R.; Kim, K.-H.; et al. The *OsERF115/AP2EREBP110* transcription factor is involved in the multiple stress tolerance to heat and drought in rice plants. *Int. J. Mol. Sci.* **2021**, *22*, 7181. [CrossRef] [PubMed]
45. He, Z.; Zhong, J.; Sun, X.; Wang, B.; Terzaghi, W.; Dai, M. The maize ABA receptors ZmPYl8, 9, and 12 facilitate plant drought resistance. *Front. Plant Sci.* **2018**, *9*, 422. [CrossRef] [PubMed]
46. Tischer, S.V.; Wunschel, C.; Papacek, M.; Kleigrewe, K.; Hofmsnn, T.; Christmann, A.; Grill, E. Combinatorial interaction network of abscisic acid receptors and coreceptors from *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA* **2017**, *114*, 10280–10285. [CrossRef]
47. Hong, C.Y.; Chao, Y.Y.; Yang, M.Y.; Cheng, S.Y.; Cho, S.C.; Kao, C.H. NaCl-induced expression of glutathione reductase in roots of rice (*Oryza sativa* L.) seedlings is mediated through hydrogen peroxide but not abscisic acid. *Plant Soil* **2009**, *320*, 103–115. [CrossRef]
48. Wang, X.S.; Zhu, H.B.; Jin, G.L.; Liu, H.L.; Wu, W.R.; Zhu, J. Genome-scale identification and analysis of LEA genes in rice (*Oryza sativa* L.). *Plant Sci.* **2007**, *172*, 414–420. [CrossRef]
49. Jin, X.; Shi, C.; Yu, C.Y.; Yamada, T.; Sacks, E.J. Determination of leaf water content by visible and near-infrared spectrometry and multivariate calibration in Miscanthus. *Front. Plant Sci.* **2017**, *8*, 721. [CrossRef] [PubMed]
50. Farooq, M.; Wahid, A.; Kobayashi, N.; Fujita, D.; Basra, S.M.A. Plant drought stress: Effects, mechanisms and management. *Agron. Sustain. Dev.* **2009**, *29*, 185–212. [CrossRef]
51. Murata, Y.; Mori, I.C.; Munemasa, S. Diverse stomatal signaling and the signal integration mechanism. *Annu. Rev. Plant Biol.* **2015**, *66*, 369–392. [CrossRef]
52. Singh, R.; Parihar, P.; Singh, S.; Mishra, R.K.; Singh, V.P.; Prasad, S.M. Reactive oxygen species signaling and stomatal movement: Current updates and future perspectives. *Redox Biol.* **2017**, *11*, 213–218. [CrossRef] [PubMed]