Article

BrPARP1, a Poly (ADP-Ribose) Polymerase Gene, Is Involved in Root Development in *Brassica rapa* under Drought Stress

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Abstract: PARP proteins are highly conserved homologs among the eukaryotic poly (ADP-ribose) polymerases. After activation, ADP-ribose polymers are synthesized on a series of ribozymes that use NAD⁺ as a substrate. PARPs participate in the regulation of various important biological processes, such as plant growth, development, and stress response. In this study, we characterized the homologue of PARP1 in *B. rapa* using RNA interference (RNAi) to reveal the underlying mechanism responding to drought stress. Bioinformatics and expression pattern analyses demonstrated that two copy numbers of PARP1 genes (BrPARP1.A03 and BrPARP1.A05) in *B. rapa* following a whole-genome triplication (WGT) event were retained compared with *Arabidopsis*, but only BrPARP1.A03 was predominantly transcribed in plant roots. Silencing of BrPARP1 could markedly promote root growth and development, probably via regulating cell division, and the transgenic *Brassica* lines showed more tolerance under drought treatment, accompanied with substantial alterations including accumulated proline contents, significantly reduced malondialdehyde, and increased antioxidative enzyme activity. In addition, the findings showed that the expression of stress-responsive genes, as well as reactive oxygen species (ROS)-scavenging related genes, was largely reinforced in the transgenic lines under drought stress. In general, these results indicated that BrPARP1 likely responds to drought stress by regulating root growth and the expression of stress-related genes to cope with adverse conditions in *B. rapa*.

Keywords: poly (ADP-ribose) polymerase; drought; reactive oxygen species; root growth; whole-genome triplication

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1. Introduction

Drought is a common abiotic stress that affects plant growth and limits crop yield and quality [1]. Long-term exposure to water-deficient conditions will affect the physiological responses of plants, such as the activities of hormone-metabolizing enzymes, the accumulation of reactive oxygen species (ROS), the opening and closing of stomata, and other characteristics, showing the phenotype of growth retardation [2–4]. ROS accumulation could cause oxidative damage to DNA and directly extract hydrogen from deoxyribose, leading to DNA single-strand and double-strand breaks, resulting in genome instability and plant aging [5]. Under drought stress, the accumulation of ROS in plants will enhance plasma membrane oxidation and protein degradation, causing oxidative damage and affecting plant growth [6]. Facing the pressure of drought, plants need complex cellular and molecular networks to establish new energy homeostasis and ensure normal growth and development. The root system is the most sensitive organ of plants to deal with

drought. When the root system feels a lack of water, it will stimulate the plant's molecular and physiological responses, adjust the morphological response, and respond to drought stress [7]. At present, in order to make the plants adapt to the changeable environments and increase the productivity of crops, breeding and biotechnology are now used to reduce the sensitivity of plants to unfavorable environments [8].

Poly (ADP-ribose) polymerase (PARP) enzymes play a key role in many cellular processes, such as DNA damage repair, maintenance of genome steadiness, and cell death [9]. Poly (ADP-ribosyl)ation (PARylation) is a reversible post-translational modification catalyzed by PARP enzymes. It participates in a series of reactions consisting of DNA damage awareness and repair, cell division and death, chromatin modification, gene transcription regulation, and stress response [10,11]. PARP uses NAD⁺ as a substrate to continuously add poly (ADP-ribose) (PAR) fragments to the amino acid receptor residues of the target protein to catalyze PARylation [9].

Three PARPs have been found in the model plant *A. thaliana*: AtPARP1 (At2g31320), AtPARP2 (At4g02390), and AtPARP3 (At5g22470), which are all located in the nucleus. AtPARP1 and AtPARP2 act as sensors of DNA damage and participate in DNA repair and stress response [12]. Experiments have shown that the double mutants of *parp1* and *parp2* in *Arabidopsis* developed more root systems under external pressure, and the main and lateral roots grow faster [13]. AtPARP3 participates in double-strand break (DSB) repair and maintains seed vigor during seed storage [14]. The activity of PARP has also been found in other plants. For instance, PARP1 and PARP2 have been found in the different species such as wheat, peas, soybean, tobacco, and corn, responding to biotic and abiotic stresses and affecting the development of leaves and roots [15–18].

In plants, PARP is the essential energy consumption under exterior stress conditions. External pressure will induce the activity of PARP, leading to the synthesis and decomposition of NAD⁺, enhancing the respiration of mitochondria and providing ATP energy, resulting in high energy consumption. A large wide variety of studies have shown that immoderate activation of PARP can cause cell death due to energy consumption [19]. Therefore, it is necessary to maintain the steady state of cell energy and reduce the consumption of NAD⁺. Chemical inhibition or silencing of PARP activity can minimize the consumption of NAD⁺ and enhance the effective utilization rate of energy, thus making plants cope better with exterior pressure [20]. Related studies proved that transgenic *Arabidopsis* with low-level PARP are more resistant to a range of abiotic stresses such as drought, robust light, and heat [21].

In the present work, we characterized a homolog of PARP1 in the major vegetable plant *B. rapa* designated as BrPARP1, and the underlying mechanism of BrPARP1 responsive to drought stress was analyzed. Hopefully this study will be of great use for genetic improvements of drought resistance in *Brassica* breeding programs.

2. Materials and Methods

2.1. Plant Material and Growth Conditions

The selfing *B. rapa* DH lines (cxl-45-05) and *A. thaliana* (Col-0) were grown in a growth chamber under the following conditions: 16 h/22 °C in light, 8 h/16 °C in darkness, and relative humidity of about 45%.

2.2. Evolution and Gene Collinearity Analysis

The genome, coding sequence, and protein sequence of each species were downloaded from Ensembl plant (<https://plants.ensembl.org/index.html>, accessed on 27 September 2020). The amino acid sequence of PARP1 protein was aligned using the MUSCLE program with default parameters. The phylogenetic tree was constructed using MEGA7 software with the maximum likelihood (ML) method and 1000 bootstrap replicates [22]. The number of PARPs was counted with evolutionary retention, and the collinearity relationship between AtPARP1 and BrPARP1 genes was visualized by Circos. The NG method was used

to calculate the synonymous mutation rate (Ks) and non-synonymous mutation rate (Ka) of the coding sequence [23].

2.3. Gene Structure Protein Motif Identification and Protein Functional Domain Analysis

To identify conserved motifs, we used MEME (<http://meme.sdsc.edu/meme/meme.html>; accessed on 27 September 2020 [24]), with the motif length set at 10–100 and motif maximum number set at 10. We analyzed the gene structure using Gene Structure Display Server (GSDS; <http://gsds.cbi.pku.edu.cn/>, accessed on 28 September 2020 [25]). Moreover, the features of genes were analyzed using EBI-Tools (<http://www.ebi.ac.uk/Tools/emboss/>, accessed on 28 September 2020). The putatively conserved function domain was analyzed by CDD (<https://www.ncbi.nlm.nih.gov/cdd>, accessed on 27 September 2020).

2.4. Subcellular Location

In order to explore the location of BrPARP1, we constructed two expression vectors for a transient expression system in *N. benthamiana*, and the transient transformation was performed as previously described elsewhere [26]. The full-length coding sequence of BrPARP1 was first cloned into pEASY-T1 vector for amplification and then subcloned into pCambia1300-GFP vector using BamHI and XbaI restriction sites. Subsequently, they were transferred to *Agrobacterium tumefaciens* (EH105) and infiltrated with the leaves using 1 mL syringes. An Olympus fluorescence microscope (DX53, Japan) was used to detect the fluorescence signal of GFP fusion protein in leaves 2 days after transformation.

2.5. Histochemical Analysis of GUS Activity

The ProBrPARP1::GUS transgenic plants were subjected to hydroponic culture, and the roots, stems, and leaves of transgenic plants grown about 3 weeks were treated with PEG6000 at 5%, 10%, 15%, and 20%. The expression pattern under osmotic stress was observed by GUS tissue staining. The staining buffer was mainly prepared with 100 μ L X-gluc stock solution and 5 mL X-gluc base solution. The samples were stained in the staining buffer at 37 °C for 12–24 h before destaining in ethanol, as previously described elsewhere [27].

2.6. Drought Stress Conditions and Phenotypic Observation

RNAi technology is a mechanism of silent gene transfer after exogenous short double-stranded RNA (dsRNA) mediated by RNAi technology to develop transgenic *B. rapa* lines [28]. Three independent transgenic *Brassica* lines—R1, R3, and R4—were selected with their expression level 20–30% of the wild-type. For drought treatment, about 4-week-old plants for transgene and WT were used by continuously withholding irrigation for 3 weeks before rewatering for recovery, and at least 10 planted pots for each treatment was used for phenotype recording with three biological replicates. In addition, 20% PEG6000 on 1/2MS medium was applied to simulate drought stress using each treatment with 10 seedling plantlets with 3 biological replicates. The root length and the number of lateral roots were counted at 6d, 8d, and 10d. Propidium iodide (PI) staining was used to stain and analyze the roots of both transgenic and WT plants [29]. The seedling roots were stained with 30 μ g/mL PI (Sigma Aldrich, St. Louis, MI, USA) for 1 min and then observed with fluorescence microscope (Carl Zeiss, Jena, Germany).

2.7. Determination of Relative Water Content (RWC)

In order to determine relative water content (RWC), we immediately weighed the leaves separated from the *B. rapa* to determine the fresh weight (FW). After determining the FW, we kept the leaves in the distillation for 12 h and weighted them as turgid weight (TW). The dry weight (DW) was recorded by drying the sample at 65 °C for 12 h. RWC was calculated as $(FW - DW)/(TW - DW) \times 100\%$ [30].

2.8. Analysis of Proline, Malondialdehyde (MDA), and NAD⁺ Content

The determination of proline content was described by Leclercq et al. (2012) [31]. In brief, a leaf sample of 0.2 g was weighted and thoroughly ground in 3 mL sulfosalicylic acid (3%), and 10 mL sulfosalicylic acid was added. After boiling in a water bath for 1 h, the sample was centrifuged after cooling. A total of 1 mL supernatant was collected and mixed with 1 mL glacial acetic acid and 1 mL acid–ninhydrin, and then heated at 100 °C for 1 h. Then, 2 mL toluene was added after cooling on ice, and the mixture was stood for 2 h after shaking. After centrifugation, the upper solution was taken, and the absorbance was measured at 520 nm by spectrophotometer. The content of proline in the sample was calculated by using toluene as a blank control.

The content of malondialdehyde (MDA) was determined according to Kong et al. (2016) [32]. A total of 1 g of fresh leaves was collected and ground in 5 mL (10%) trichloroacetic acid; then, the mixture was centrifuged at 3500× g for 10 min. The supernatant (1 mL) was mixed with an equal amount of 0.6% thiobarbituric acid and then placed in a water bath (100 °C, 20 min). The supernatant was centrifuged and measured for the absorbance at 450, 532, and 600 nm.

The determination of NAD⁺ was referred to the Greiss Company's instructions. Briefly, about 0.1 g fresh leaves were collected and added with 1 mL extraction buffer, and then the sample was ground in an ice bath followed by incubation at 95 °C for 5 min. The sample was then immediately placed on ice bath for 5 min and centrifuged at 12,000 rpm at 4 °C for 10 min. The 500 µL supernatant was taken out and added with 500 µL V1 extract buffer and then centrifuged at 12,000 rpm at 4 °C for 5 min. A total of 100 µL supernatant was taken out and analyzed with the spectrophotometer at 450 nm.

2.9. Quantitative Real-Time PCR Analysis

Total RNA extractions, isolated from *B. rapa*, were performed with Plant Total RNA Isolation Kit Plus (Foregene, Chengtu, China), using 100 mg of fresh leaf tissue. The purified RNA was measured with an ultra-micro spectrophotometer (Nanodrop 2000). The cDNA was synthesized by HiFiScript cDNA synthesis Kit (CWBio, Beijing, China). The Lunar[®] Universal qPCR Master Mix (NEB, Beijing, China) and Light Cycler 480 system were used for qRT-PCR analysis. The PCR conditions were 94 °C for 30 s, 40 cycles at 94 °C for 10 s, and 58 °C for 30 s, followed by a melting curve to determine the specificity of the amplification. Relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method [33], and β -actin was used as internal control with three biological replicates. All the primers used are listed in Table S1.

2.10. Statistical Analysis

The experimental data involved in this experiment have at least 3 independent biological and technical replicates. All data graphs were drawn and analyzed using GraphPad Prism 5, and one-way ANOVA was used to analyze the significance of differences between the experimental and the control with the *t*-test applied. ** $p < 0.01$ indicates that the difference between the data was very significant, whereas * $p < 0.05$ indicates that the difference was significant.

3. Results

3.1. Conservation of BrPARP Genes following Whole-Genome Triplication Event in *B. rapa*

Compared with *A. thaliana*, evolutionarily, the *Brassica* species has experienced an additional whole-genome triplication (WGT) event, and the duplicated genes were likely retained or lost due to the successive genomic rearrangements [34]. Thus, we constructed the phylogenetic relationship, gene structure, and functional domain of the PARP protein in three typical diploid *Brassicaceae* species, namely, *A. thaliana*, *B. rapa*, and *B. oleracea*, and analyzed the copy number of the PARP genes retained in their genome (Figure 1A). The results showed that the putatively duplicated genes PARP2 and PARP3 were both retained with single copy number in *B. rapa* and *B. oleracea*, indicating a loss of function

probably induced by genomic rearrangements, but *PARP1* gene retained redundancy with two copies in *B. rapa* and three in *B. oleracea* after the WGT event (Figure 1A). We used the Circos to analyze the *AtPARP1* and two *BrPARP1* genes for collinearity, which showed the retained *BrPARP1* genes were located on chromosome 3 and 5 (respectively, *BrPARP1.A03* and *BrPARP1.A05*) in *B. rapa* (Figure 1B). Further, the non-synonymous/synonymous mutation rate (K_a/K_s) between gene pairs was calculated (Figure 1C), and the calculated K_a/K_s ratio between *BrPARP1* and *AtPARP1* gene pairs was found to be about 0.29–0.43, which was generally believed to experience lower selection pressure and should be functionally conserved during evolution. In addition, we analyzed the *PARP1* gene structure and protein conserved domains between *B. rapa*, *B. oleracea*, and *A. thaliana* (Figure 1D). The results showed that the structure and conserved domain between *BrPARP1.A03* and *BrPARP1.A05* were highly similar, inferring both *BrPARP1* genes might be functionally redundant in *B. rapa*.

Figure 1. *PARP* genes following WGT in brassicas. (A) The number of *PARP* genes copies retained in *Arabidopsis* and *Brassica* species. The phylogenetic tree resulting from the evolution of species. (B) Collinear correlations of *BrPARP1* genes and neighboring genes in the *A. thaliana* and *B. rapa* genomes. The *B. rapa* and *A. thaliana* chromosomes are colored in accordance with the inferred ancestral chromosomes following a set up convention. The lines of *BrPARP1* genes are red and lost genes are gray. The figure was created using Circos software. (C) The K_a/K_s value of *B. rapa* and *A. thaliana* *PARP1* orthologous gene pair and paralogous gene pair. (D) Gene structures and conserved motifs of *PARP1*. The unrooted phylogenetic tree resulting from the full-length amino acid alignment of all of the *PARP1* proteins. The tree was constructed the usage of maximum likelihood (ML) and bootstrap values calculated with 1000 replications using MEGA7.

3.2. Expression Pattern and Subcellular Localization of *BrPARP1* in *B. rapa*

The expression level of two *BrPARP1* copies (*BrPARP1.A03* and *BrPARP1.A05*) was verified in different tissues in *B. rapa* (Figure 2A), and the results showed that transcription

level of *BrPARP1.A03* (ID: Bra000883) was predominantly higher than that of *BrPARP1.A05* (ID: Bra018555) in the assayed tissues, as well as the highest expression level detected in roots, followed by stems, leaves, and flowers, which indicated that *BrPARP1.A03* played main functions in *B. rapa*. In addition, transient expression analysis of the *PARP1.A03*–GFP fusion protein in *N.benthamiana* leaf cells confirmed that the fusion protein is located in the nucleus (Figure 2B), which echoes the role of PARP in DNA repair and transcriptional regulation [12].

Figure 2. The expression of *BrPARP1* and subcellular location. (A) Analysis of *BrPARP1* for tissue-specific expression. The Actin gene was used as a reference. (B) Subcellular localization of the fused 35S::*BrPARP1*-GFP in *N. benthamiana* leaf cells. The 35S::GFP construct as the control. Bar = 50 μ m. (C) Histochemical staining in transgenic *A. thaliana* plants. GUS stains analysis proCaMV35S::GUS transgenic *A. thaliana* plants. GUS stains analysis pro*BrPARP1*::GUS transgenic *A. thaliana* plants after 0%, 5%, 10%, 15%, and 20% PEG6000 treatment. Bar = 5 mm.

In order to verify the expression pattern of *BrPARP1.A03* in response to drought, we used PEG6000 to simulate drought stress. The pro*BrPARP1*::GUS transgenic lines were obtained, and different concentrations of PEG6000 (0%, 5%, 10%, 15%, 20%) were used for stress treatment (Figure 2C). The results showed that *BrPARP1* promoter was not easily activated under mild drought treatments (5–10% PEG6000) in the seedling leaves, but sensitively detected in roots. Especially under harsher treatments (15% to 20% PEG6000), GUS activity driven by *BrPARP1* promoter gradually increased in all tissues treated with PEG6000.

3.3. Silencing of BrPARP1 Enhanced Plant Tolerance in *B. rapa* under Drought Stress

To explore the response of BrPARP1 gene under drought stress, we obtained the transgenic lines via RNA-induced gene silencing in *B. rapa*. About 4-week-old plants were used for drought treatment by withholding irrigation for 3 weeks; the data showed the transgenic *Brassica* lines exhibited a better survival ability under drought treatments, and after rewatering, the transgenic plants could better recover their growth ability (Figure 3A). The leaf relative water contents (RWC) showed less difference between transgenic lines and wild-type under normal growth conditions, but a significant difference was observed under drought treatments (Figure 3B). In addition, more proline accumulated in the leaves of transgenic lines (Figure 3C), as an osmotic protection substance to reduce potential damages to plants due to drought and water loss [35]. Reactive oxygen species (ROS) were also analyzed after exposure to drought stress, and the results showed that transgenic lines suffered less oxidative damage compared with WT (Figure 3D). Malondialdehyde (MDA) was generally considered as an indicator of oxidative damage to plant cell membranes under stresses [36,37], and the MDA content was thus determined, which was significantly lower in transgenic lines compared with WT (Figure 3E). Nicotinamide adenine dinucleotide (NAD⁺) is a vital pyridine nucleotide, and its depletion may occur in response to excessive DNA damage caused by free radicals. Our data showed that the transgenic lines maintained a higher NAD⁺ level compared with WT under drought treatments (Figure 3F), which was probably owing to reduced PARP activity [38,39]. Overall, the outcomes indicated that silencing of BrPARP1 could enhance the tolerance of plants to drought stress and enlarge the survival rate of plants under adverse environmental conditions.

Figure 3. (A) Phenotype analysis of WT and RNAi-BrPARP1 during dehydration treatment. Three RNAi-BrPARP1 transgenic lines and WT were treated with withholding irrigation at the seedling stage for about 3 weeks. (B) Relative water content of detached leaves of RNAi-BrPARP1 and WT plants. (C) Proline content of RNAi-BrPARP1 and WT plants under normal conditions and after water deprivation. (D) ROS content of RNAi-BrPARP1 and WT plants. (E) MDA content of RNAi-BrPARP1 and WT plants under normal conditions and after water shortage. (F) NAD⁺ content of RNAi-BrPARP1 and WT plants under normal conditions and after water shortage. Values were means $\pm$ se of biological replicates ($n > 3$). (** $p < 0.01$, * $p < 0.05$).

3.4. BrPARP1 Regulated Root Developments in *B. rapa* under Drought Stress

The morphology of plant roots plays a vital role in plant growth and environmental adaptability [40]. We analyzed the root growth via silencing BrPARP1 in the transgenic plants under drought conditions. The results showed that compared with the WT, the transgenic lines had more developed root systems under both normal conditions and drought treatment (Figure 4A), and silencing of BrPARP1 could accelerate main root growth and lateral root developments (Figure 4B–D). In addition, we found that the genes (*LOX1*, *CEG*, and *TIR1*) related to lateral root development were significantly upregulated in transgenic lines, which indicated that silencing of BrPARP1 could promote root development and enhance drought resistance in *B. rapa*.

Figure 4. Analysis of the effect of BrPARP1 on root growth. (A) Comparison of the root system of WT and RNAi-BrPARP1 transgenic plants under normal conditions and after 20% PEG6000 treatment. Bar = 10 mm. (B) Principal root length analysis of WT and RNAi-BrPARP1 transgenic plants at 6 days, 8 days, and 10 days. (C) Lateral root length analysis of WT and RNAi-BrPARP1 transgenic plants at 6 days, 8 days, and 10 days. (D) Lateral root number analysis of WT and RNAi-BrPARP1 transgenic plants at 6 days, 8 days, and 10 days. (E) Analysis expression of lateral root-related genes. *LOX1*: Lectin-like oxidized low-density lipoprotein receptor-1; *CEG*: Clusters of Essential Genes; *TIR1*: Transport Inhibitor Response 1 (** $p < 0.01$, * $p < 0.05$).

3.5. BrPARP1 Affected Root Cell Division in *B. rapa*

Root growth involves cell division in the meristematic zone and cell elongation in the elongation zone [41]. We found that in comparison with the wild-type, the transgenic lines had more layers of cells in root meristematic zone (Figure 5A), and the length of the principal root meristem was longer (Figure 5B), the continuous cell division and differentiation within root tips promoted its growth and development, and the difference in the length of meristem layer revealed the rapid root growth of transgenic plants. In addition,

we examined the transcription of key genes related to cell cycle control (Figure 5C), and the results showed that the expression of mitotic regulators such as type B cyclin (*CycB1;1*) increased significantly, but the transcription level of the kinase gene *CHK2* involved in apoptosis was obviously reduced. At the same time, the expression of nuclear replication-related gene *TOP6B* was significantly reduced, and the transcription level of ATM that regulates the cell cycle kinase was also downregulated in the transgenic plants. These results indicated that silencing of BrPARP can enhance cell division via partially inhibiting root nuclear endoreduplication in *B. rapa*.

Figure 5. Analysis of BrPARP1 affecting root growth. (A) Propidium iodide-stained root meristems of WT and RNAi-BrPARP1 plants. Bar = 100 μm. (B) Principal root meristem length of WT and RNAi-BrPARP1 plants. (C) Expression level analysis of root growth transition marker genes. *CYCB1.1*: 1 Cyclin B Transcripts; *CHK2*: Checkpoint kinase2; *TOP6B*: Topoisomerase VI; *ATM*: Ataxia Telangiectasia Mutated. (** $p < 0.01$, * $p < 0.05$).

3.6. BrPARP1 Regulated Expression of Stress-Related Genes in *B. rapa* under Drought Stress

In order to clarify the BrPARP1-mediated mechanisms regarding on the enhanced drought tolerance of transgenic *B. rapa* lines, we selected several key transcription factors, which were closely related to drought stress, namely, *RD26*, *SOS1*, *CBL1*, *RHL2*, and *DREB2A* (Figure 6). These results showed that these assayed genes were significantly upregulated under drought treatment in the transgenic lines compared to the wild-type.

These results indicated that BrPARP1 may respond to drought stress by cooperating with stress-related genes, so as to better cope with external pressure.

Figure 6. Expression analysis of stress-related genes in *B. rapa* under drought treatment. RD26: RESPONSIVE TO DESICCATION 26; SOS1: STRESS OVERLY SENSITIVE 1; CBL1: CALCINEURIN B-LIKE PROTEIN 1; RHL2: ROOT HAIRLESS 2; DREB2A: DEHYDRATION RESPONSIVE ELEMENT-BINDING PROTEIN2A. (** $p < 0.01$, * $p < 0.05$).

4. Discussion

4.1. BrPARP1 Probably Functions as a Single Copy Gene in *B. rapa* during Evolution

Poly (ADP-ribose) polymerase (PARP) is a post-translational modification enzyme in organisms. It belongs to the PARP family and has the function of catalyzing the formation of linear or branched poly ADP-ribose (PAR) polymer from ADP ribose. It is highly conservative in evolution. In the process of evolution, whole-genome duplications (WGD) do not solely cause genetic abundance but may also produce a large amount of genetic variation [42]. In *Brassica* crops, it has experienced an additional WGT event, which has an important effect on the richness and functional diversity of the species [43]. However, some studies had reported that there is a set of genes in *A. thaliana* and *O. sativa*, existing in the form of a single copy after external pressure selection [44]. The existence of single-copy genes in eukaryotes may be due to the basic functions that are often highly conserved, and the expression of single-copy genes in tissues will be higher, with higher conservation [45,46]. In order to explore the effects on the copy number of *PARP* gene after WGT event in *Brassica*, this study selected *A. thaliana*, *B. oleracea*, and *B. rapa*. Bioinformatics analysis showed that *PARP2* and *PARP3* retained one copy number in both *B. rapa* and *B. oleracea*, but *B. rapa* and *B. oleracea* retained two and three copies of the *PARP1* gene after the WGT event, respectively (Figure 1A), while *PARPs* mostly retain single copy form during the evolutionary process, which means that despite the replication event, the gene will still exist as a single-copy form. In this study, the *AtPARP1* and *BrPARP1* gene pairs were analyzed for collinearity (Figure 1B). The results showed that after WGT, the *BrPARP1* gene was duplicated into three copies, and one copy was lost in the evolutionary process, leaving *BrPARP1.A03* and *BrPARP1.A05* in the subgenome. Analysis of the gene structure, conserved domains (Figure 1D), and gene transcription level (Figure 2A) of *BrPARP1.A03* and *BrPARP1.A05* showed that *BrPARP1.A03* is more conservative in evolution. Therefore, after WGT events, the duplicated *BrPARP1* gene may have undergone gene loss, and mainly functions in the form of a single copy. The communication between nuclear genes and different organelles is strictly regulated and plays an important role in maintaining the relative balance of proteins encoded between organelles [47]. The increase in gene copy

number caused by WGD events may lead to the relative imbalance of protein interaction between organelles, which may have negative effects on plants [48]. It is possible that increase in copies of genes may be deleterious in some cases, and thus these genes may exist mainly in the form of a single copy. The WGT event in *B. rapa* has witnessed the power of gene evolution, which has enlightened significance for the functional analysis of multi-copy genes.

4.2. *BrPARP1* Likely Regulated Root Development Responsive to Drought Stress

Drought stress hinders cell division and elongation, ultimately reducing crop yields and causing economic losses [49]. Plants that maintain root growth under drought conditions have a higher survival rate. Therefore, this study explored the phenotypic differences between RNAi-*BrPARP1* transgenic and wild-type plants under the drought treatment for about 3 weeks and 20% PEG6000 osmotic stress conditions. Under normal conditions, the RNAi-*BrPARP1* transgenic line and the wild-type plants showed similar growth ability. When withholding irrigation for about 3 weeks, the RNAi-*BrPARP1* transgenic lines were less wilted compared with the wild-type plants. After 3 days of rewatering, most of the RNAi-*BrPARP1* transgenic lines can survive and regain vitality, but the WT plants cannot recover themselves. Under the 20% PEG6000 osmotic stress, RNAi-*BrPARP1* transgenic plants have a more developed root system, and the main root length and the number of lateral roots increased significantly. Therefore, we used propidium iodide (PI) staining method to compare the staining of RNAi-*BrPARP1* transgenic plants and wild-type roots. The results showed that the RNAi-*BrPARP1* transgenic plants had more cells in the root meristem, which was consistent with the root phenotype observed. The response of plants to stress is often the result of multi-gene synergy. The expression level of transcripts of related stress genes is an important basis for verifying plant resistance. It has been reported that *RD26* and transcription factor *DREB2A* are significantly upregulated in *B. juncea* L under water deficit, playing an important role in improving tolerance to drought stress [50]. The high transcription levels of *SOS1* and *CBL1* can make plants more drought-tolerant and salt-tolerant, helping plants to be better able to cope with external pressures [51,52]. In this study, the stress-related marker genes *RD26*, *SOS1*, *CBL1*, *RHL2*, and *DREB2A* were thus selected for real-time quantitative PCR analysis. The results showed that these stress genes were all upregulated in RNAi-*BrPARP1* transgenic plants compared with the wild-type.

In summary, we found that after RNA interference with *BrPARP1* expression, RNAi plants had a more developed root system, which improved the water absorption capacity of plants, thereby showing a higher stress tolerance behavior. Previous studies have shown that root elongation helps plants respond to drought stress [53]. Nicotinamide adenine dinucleotide (NAD⁺) is an important pyridine nucleotide that plays an important cofactor and substrate role in a series of key cellular processes such as oxidative phosphorylation, ATP production, DNA repair, and epigenetic regulation of gene expression [54]. Studies have shown that increasing NAD⁺ levels can greatly reduce oxidative cell damage in metabolic tissues. After oxidative damage, inhibiting *PARP* activity can maintain NAD⁺ and ATP levels and prevent cell lysis [55]. These data support the previous results that the reduction of *PARP* activity can improve the tolerance of abiotic stress, as well as confirming the potential application of *PARP* gene in the induction of plant tolerance during breeding program [20].

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/horticulturae8010078/s1>, Table S1: Primer information sequence.

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