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Arabidopsis poly(ADP-ribose) glycohydrolase 1 is required for drought, osmotic and oxidative stress responses

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ABSTRACT

Poly(ADP-ribosyl)ation is a post-translational protein modification that plays important roles in many cellular processes in mammalian systems. Emerging evidence indicates that poly(ADP-ribosyl)ation is also involved in plant growth, development, and stress responses. In the present study, we used genetic mutant *parg1-3* and transgenic *PARG1*-overexpressing *Arabidopsis* plants to examine the role of poly(ADP-ribose) glycohydrolase1 (PARG1) in abiotic stress resistance. Osmotic (mannitol treatment) or oxidative [methyl viologen (MV) treatment] stress reduced germination rates of the *parg1-3* seeds compared with wild type seeds. The *parg1-3* plants showed reduced tolerance to drought (withholding water), osmotic, and oxidative stress, as well as increased levels of cell damage under osmotic and oxidative stress and reduced survival under drought stress when compared with the wild type plants. Stomata of the *parg1-3* plants failed to close under drought stress conditions. The expression level of oxidative stress-related genes *AtAox1* and *AtApx2* in the *parg1-3* plants was reduced after MV treatment. However, when *PARG1* was overexpressed in the *parg1-3* mutant and the wild type Col-0 background, similar phenotypical changes to wild type were noted in response to drought, osmotic, or oxidative stress. These results suggest a function for PARG1 in abiotic stress responses in Arabidopsis.

1. Introduction

During their lifespan, plants encounter many unfavorable environmental conditions, such as drought, salinity, and oxidative stress, which can adversely affect their growth and development [1]. To cope with abiotic stress, plants invoke multiple complicated and precisely regulated physiological and molecular networks, which are only now becoming understood through a combination of physiological, biochemical, molecular, genetic and genomics studies [2–6]. These responses of plants to environmental stress are now recognized to occur through altered expression of many abiotic stress-related genes, many of which have great potential for crop improvement [7–11].

Recent studies have demonstrated that post-translational modifications of some regulatory proteins can modulate plant responses to abiotic stresses [12–15]. Poly(ADP-ribosyl)ation is an immediate, but transient, post-translational protein modification. This reaction is achieved by poly(ADP-ribose) polymerases (PARPs), which catalyze the transfer of ADP-ribose moieties from the substrate nicotinamide adenine dinucleotide (NAD^+) to target proteins to form poly(ADP-ribose) polymers [16,17]. By contrast, poly(ADP-ribose) glycohydrolases (PARGs) degrade poly(ADP-ribose) polymers [16,17]. Proteins modified by poly(ADP-ribosyl)ation are involved in a wide range of cellular processes in animal systems, including chromatin decondensation, centrosome duplication, and telomere integrity, as well as cell division, transcription, DNA repair, cell survival, and death [17–23].

Increasing evidence now indicates that poly(ADP-ribosyl)ation is also one of the important regulatory mechanisms that modulate plant responses to various abiotic stresses. The first line of evidence came from experiments with cultured soybean and tobacco suspension cells that were protected from programmed cell death triggered by H_2O_2 or heat shock by the addition of PARP inhibitors [24,25]. Later studies showed that DNA damage induced by ionizing radiation activates a rapid and massive expression of *PARP1* and *PARP2* genes in all Arabidopsis tissues, whereas the accumulation of *PARP2* transcripts is preferentially induced by dehydration and cadmium stress [26]. Further functional analysis revealed an inhibition of cell death and conferral of more tolerance to a broad range of abiotic stresses, such as high light intensity, drought, and heat stress, when PARP activity was reduced by means of chemical inhibitors or by gene silencing [27]. Similarly, reduction

Abbreviations: CaMV, cauliflower mosaic virus; MV, methyl viologen; NAD^+, nicotinamide adenine dinucleotide; PARP, poly(ADP-ribose) polymerases; PARG, poly(ADP-ribose) glycohydrolases.

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of PARP2 levels by RNAi-mediated downregulation in transgenic Arabidopsis and oilseed rape plants resulted in greater resistance to various abiotic stresses, including drought stress, in laboratory and greenhouse experiments, but had no significant effect on growth, development, and seed production [28]. This increased stress tolerance was initially attributed to maintenance of energy homeostasis due to reduced NAD^+ consumption or increased levels of cyclic ADP-ribose, but microarray-based gene expression profiling revealed an up-regulation of a large set of abscisic acid (ABA)-responsive genes in *PARP2*-deficient plants [27–29]. Recent studies have also implicated PARP in plant responses to pathogen infection, as the induction of innate immune responses (e.g., callose deposition, lignin deposition, and phenylalanine ammonia lyase activity) by treatment with two well-known microbe-associated molecular patterns, flg22 and elf18, which can be blocked by PARP inhibitors [30,31].

In contrast, little is known about the functions of PARGs in plants. Recently, *PARG1* (or At2g31870, also known as *TEJ*) was implicated as a regulator of the circadian oscillator because mutation of *PARG1* in Arabidopsis affected the clock-controlled transcription of genes and altered the timing of photoperiod-dependent transition from vegetative growth to flowering [32]. Expression of putative *PARG* genes including *PARG1* was also up-regulated in response to oxidative stress caused by methyl viologen (MV) [33]. Functional analysis using T-DNA insertion lines indicated that mutations in both *PARG1* and another putative *PARG* gene (At2g31865) accelerated the onset of disease symptoms caused by infection with *Botrytis cinerea* [31]. Therefore, like PARPs, PARGs also appear to have diverse functions in plant biotic and abiotic stress responses.

In our study on the function of PARG1 in disease resistance response, we occasionally observed that plants of a *parg1* mutant line suffered drought stress while the wild type plants grew normally in an accident that all Arabidopsis plants grown in a growth room were not watered for a period of 4-days. In the present study, we thus examined in detail the possible function of PARG1 in abiotic stress tolerance in Arabidopsis using genetic mutant *parg1-3* and transgenic *PARG1*-overexpressing plants. Our results indicate that *PARG1* is required for tolerance to drought, osmotic and oxidative stress in Arabidopsis and thus suggest an important role for PARGs in abiotic stress response in plants.

2. Materials and methods

2.1. Plant materials and growth conditions

Seeds of wild type (ecotypes Col-0 and Ws-0) and a T-DNA insertion line (FLAG315E11) were obtained from the *Arabidopsis thaliana* Resource Centre at Ohio State University, USA, and the *Arabidopsis thaliana* Resource Centre for Genomics at the Versailles Genetics and Plant Breeding Laboratory, France, respectively. All Arabidopsis plants were grown in soil or grown on a 1/2 Murashige and Skoog (MS) medium containing 1% sucrose and 0.8% agar in a growth room under fluorescent lighting ($150\,\mu E\,m^2\,s^{-1}$) at $22 \pm 2\,^{\circ}C$ with 60% relative humidity and a 12 h light/12 h dark cycle.

2.2. Identification of the parg1-3 mutant line

Homozygous plants from the FLAG315E11 line were obtained by polymerase chain reaction (PCR)-based genotyping using a pair of gene-specific primers 315E11-LP (TAC TCT CGA GCC ATC TGC TTC) and 315E11-RP (GTG AAC TCC CAA TGG AGA CTG) along with a T-DNA primer F-LB4 (CGT GTG CCA GGT GCC CAC GGA ATA GT). Seeds from homozygous plants were used for all experiments.

2.3. Generation of transgenic overexpression lines

To generate transgenic overexpression lines in *parg1-3* or wild type Col-0 background, the coding region of the *PARG1* gene was amplified by RT-PCR using a pair of gene-specific primers PARG1-orf-1F (ATA GAA TTC ATG GAG AAT CGC GAA GAT CT) (EcoRI site underlined) and PARG1-orf-1R (GCA GTC GAC TCA AGG CGG CTG CAT AGC TT) (SalI site underlined). The amplified coding region was cloned into pUCm-T vector by T/A cloning, yielding pUCm-PARG1-1 plasmid, confirmed by sequencing from both directions. The coding region was released from the pUCm-PARG1-1 plasmid by digestion with EcoRI/SalI and then inserted into the EcoRI/SalI sites of a binary vector pCAMBIA 99-1 under control of the cauliflower mosaic virus (CaMV) 35S promoter in the sense orientation, thus yielding pCAMBIA991-PARG1-1 plasmid. This recombinant plasmid was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation using a GENE PULSER II Electroporation System (Bio-Rad Laboratories, USA).

Transformation was performed using the floral dip method as described previously [34]. Seeds from transformed plants (T0) were harvested and screened on 1/2 MS medium containing hygromycin (Hgr) at 30 μg/ml. Transformants of the T1 generation were selected and self-pollinated. The progeny of the T1 transformants were observed on selective medium and transgenic lines with 3:1 (Hgr-resistant/Hgr-sensitive) segregating ratio were selected and transferred to soil for self-pollination. Progeny of the individual T2 plants were observed on selective medium and those lines whose seedlings showed Hgr resistance were selected as homozygous lines and used for further studies.

2.4. Seed germination assays

Seeds were surface-sterilized and plated on 1/2 MS medium supplemented with 400 mM mannitol or 10 μM MV (Sigma, USA) or with same volume of water (control). The plated seeds were incubated at 4 °C for 48 h to synchronize germination and the seed germination (emergence of radicals) was scored every two days. Experiments were independently repeated at least three times.

2.5. Drought and oxidative stress treatments

Drought stress treatment was performed by withholding water for 2 weeks from soil-grown four-week-old plants. After the 2 week drought period, the plants were re-watered and the numbers of plants that continued to grow were recorded to calculate the survival rate [35].

For osmotic stress treatment, three-week-old plants grown on 1/2 MS medium were removed and transferred into water for 20 h. The plants were then transferred into solutions supplemented with 0 mM or 500 mM mannitol for 4 h. Tolerance to osmotic stress was evaluated by quantification of electrolyte leakage after stress treatment [36]. Briefly, initial conductivity of the bathing solution was measured using a DDS-IIAT type conductivity 510 meter. The samples in the bathing solutions were then boiled for 5 min and volumes of the bathing solution were brought up to the initial volumes, followed by measurement of the total conductivity. The percentage of electrolyte leakage was calculated as 100 × (initial conductivity of the test samples)/(total conductivity after boiling).

For oxidative stress treatment, four-week-old plants grown in soil were treated by foliar spraying with 50 μM MV and symptoms were observed. Alternatively, leaves detached from four-week-old plants were floated on 1/2 MS liquid medium containing 1 μM MV, and oxidative stress response was evaluated by measuring the chlorophyll content of the leaf tissues according to the method described previously [37]. Chlorophyll was extracted with 95% ethanol overnight, and the content was determined spec-

trophotometrically. Chlorophyll content was calculated according to the formula Chl (A + B) = $5.24A_{664} + 22.24A_{648}$, where Chl is the chlorophyll concentration in micrograms per milliliter and *A* is the absorption.

All experiments were independently repeated at least three times.

2.6. Measurement of stomatal apertures

For measuring stomatal apertures in response to drought stress, three-week-old plants grown in soil were subjected to drought treatments by withholding water for 2 weeks. The abaxial epidermis was peeled from rosette leaves of stressed plants and stomatal apertures were measured according to the method as described previously [38]. At least 20 stomatal apertures on the fourth leaves of 10 individual plants were measured in each experiment and all experiments were independently repeated at least three times.

2.7. Analysis of gene expression by RT-PCR

For expression of *PARG1* in *parg1-3* mutant and transgenic overexpression lines, leaf samples were collected from four-week-old soil-grown plants grown. For expression of *PARG1* in response to oxidative stress, leaves on four-week-old wild type and *parg1-3* mutant plants were sprayed with 25 μM MV and leaf samples were collected at 0 h, 12 h and 24 h after spraying. Total RNA was extracted using TRIZOL reagent (Invitrogen, Shanghai, China) according to the manufacturer's instructions. A 500 ng sample of total RNA was used to synthesize first strand cDNAs using an RNA PCR Kit (AMV) Ver. 3.0 (Takara, Dalian, China) according to the supplier's instructions. Expression of *PARG1* was analyzed by RT-PCR with primers of PARG1-rt-1F (CGC ATT TCT CTT TGA CGA ATT GAT TG) and PARG1-rt-1R (CAA TTA ACC CAA AAG AGT GAA CCT). Primers used for analysis of expression of oxidative stress-related genes were as follows: AOX1A-1F, CGT GTG AAG CGT ATA AAG ACG ACA A; AOX1A-1R, CCA AGT ATG GCT TAA GCA GAG GTG A; AtApx1-1F, CTG TTG AGA AGT GCA GGA GGA AGC; AtApx1-1R, CAT GTG GGC CTC AGC GTA ATC AGC; AtApx2-1F, TGC TGT TGA GAT CAC TGG AGG AC; AtApx2-1R, GAT GAG CTT CCG TAT AGT CTT CG; AtFSD1-1F, AGT TCA ATG CTG CTG CAG CCA CTC; and AtFSD1-1R, GCA GAA CTC ACT GTC ACT GAA GTC. PCR conditions were set as 94 °C 30 s, 55–60 °C 30 s and 72 °C 50 s for 33 cycles, followed by 5 min of final extension at 72 °C. PCR products were electrophoresed on a 2.0% agarose gel.

2.8. Statistical analysis

All data were statistically analyzed using *t*-tests.

3. Results

3.1. Characterization of parg1-3 mutant and transgenic overexpression lines

The Arabidopsis *PARG1* gene (At2g31870) consists of 11 exons and 10 introns (Fig. 1a) and this annotation for exon/intron organization of the *PARG1* gene is confirmed by two full-length cDNAs (AF394690 and AK222165) in the GenBank database. Two T-DNA insertion lines, SALK_147805 and SALK_116086, were previously identified and designated *parg1-1* and *parg1-2*, respectively (Fig. 1a) [31]. The *parg1-1* and *parg1-2* lines contain T-DNA insertions in the eighth and ninth introns, respectively, which are very close to the stop codon of the *PARG1* gene. In this study, we identified a mutant line, designated *parg1-3*, with a T-DNA insertion in the third exon of the *PARG1* gene, from a France PublicLine T-DNA insertion population in Ws-0 background. RT-PCR analysis revealed no *PARG1* gene transcript in homozygous plants of the *parg1-3* mutant line under normal conditions (Fig. 1b). Transgenic lines overexpressing the *PARG1* gene, driven by CaMV 35S promoter, were also generated in the *parg1-3* mutant and wild type Col-0 background. Homozygous lines with a single copy of the *PARG1* transgene were obtained through antibiotic resistance segregation screenings. Two independent transgenic lines overexpressing *PARG1* gene in *parg1-3* mutant background (*PARG1*-OE1/*parg1-3* and *PARG1*-OE2/*parg1-3*) or wild type Col-0 background (*PARG1*-OE1/Col-0 and *PARG1*-OE2/Col-0) were selected for further studies. RT-PCR analysis showed that expression levels of the *PARG1* gene were much higher in transgenic overexpression lines in *parga1-3* mutant and wild type Col-0 background than in the corresponding wild type Ws-0 and Col-0 plants (Fig. 1b and supplementary Fig. 1). Therefore, the *parg1-3* mutant line and transgenic overexpression lines (*PARG1*-OE1/*parg1-3*, *PARG1*-OE2/*parg1-3*, *PARG1*-OE1/Col-0 and *PARG1*-OE2/Col-0) were used for functional analysis.

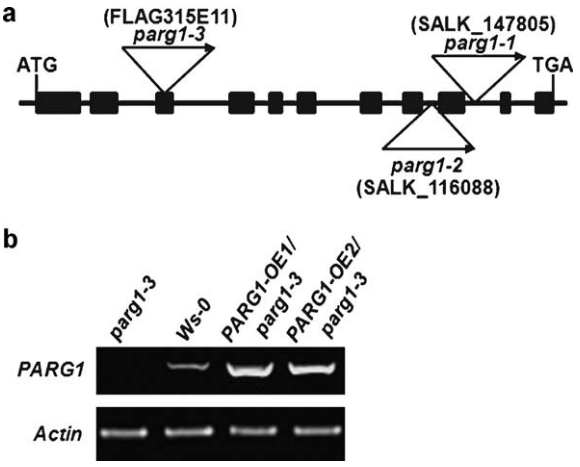

Fig. 1. Identification of *parg1-3* mutant and transgenic overexpression lines. (a) Exon/intron organization of the *PARG1* gene and the T-DNA insertion lines for *parg1* mutants. Filled boxes indicate exons while solid lines indicate introns of the *PARG1* gene. Triangles with arrows indicate the insertion sites and the orientation of the T-DNA in *parg1* lines. (b) Confirmation of *PARG1* expression in *parg1-3* and transgenic overexpression lines *PARG1-OE1/parg1-3* and *PARG1-OE2/parg1-3*. Actin was used as an internal control in (b).

3.2. Mutation in PARG1 attenuated osmotic stress tolerance

We first examined and compared the phenotype of *parg1-3* mutant and transgenic overexpression lines with respect to responses to osmotic stress mimicked by mannitol treatment. On MS medium without mannitol supplementation, germination of seeds from *parg1-3* mutant and transgenic overexpression lines was similar to that of wild type Ws-0 seeds (Fig. 2a, left). In contrast, germination of *parg1-3* mutant seeds was much poorer than that of the Ws-0 seeds on MS medium supplemented with 400 mM mannitol (Fig. 2a, right and b). When three-week-old plants were placed in 500 mM mannitol solution, electrolyte leakage from *parg1-3* plant tissues was much higher than that from wild type plant tissues (Fig. 2c), indicating an enhanced sensitivity to osmotic stress.

In the transgenic overexpression lines (*PARG1*-OE1/*parg1-3*, *PARG1*-OE2/*parg1-3*, *PARG1*-OE1/Col-0 and *PARG1*-OE2/Col-0), seed germination was unaffected by treatment with MS medium supplemented with 400 mM mannitol (Fig. 2b and supplementary Fig. 2a). The electrolyte leakage levels in three-week-old transgenic overexpression plants were also comparable to those in the wild type Ws-0 and Col-0 under osmotic stress induced by mannitol treatment (Fig. 2c and supplementary Fig. 2b). Thus, loss of *PARG1* function can attenuate osmotic stress tolerance while overexpression of *PARG1* does not improve osmotic stress tolerance. Taken together,

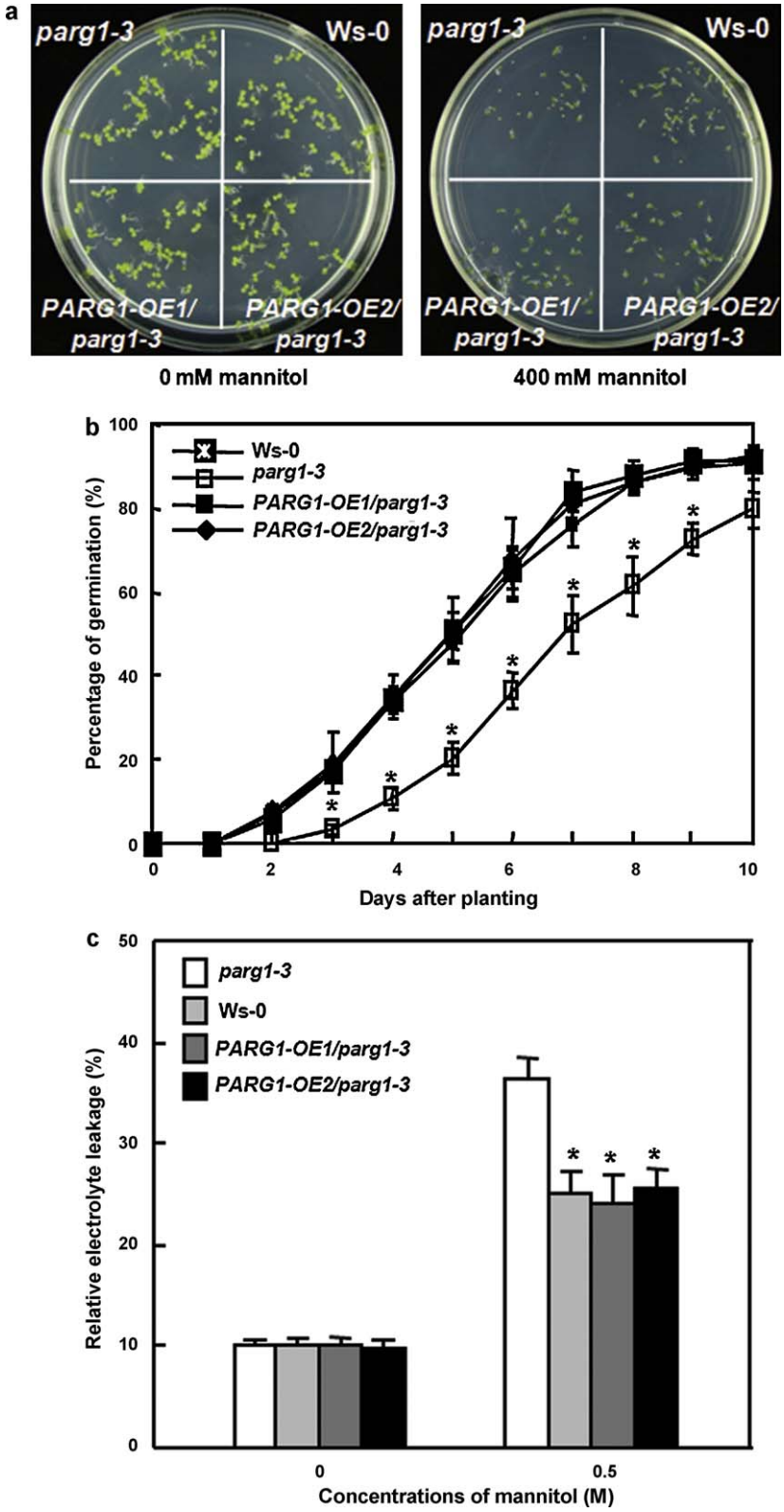

Fig. 2. Reduced tolerance to osmotic stress in *parg1-3* plants. (a) Photographs were taken on the 8th day after planting on 1/2 MS medium containing 400 mM mannitol. (b) Seed germination rates. (c) Electrolyte leakage after treatment with mannitol. Three-week-old plants were taken from MS plates and placed into 0 mM (as control) and 500 mM mannitol solution for 4 h. Data presented in (b) and (c) are the means and standard errors from three independent experiments. Asterisks above the open squares (b) or columns (c) indicate significant difference at $p = 0.05$ level by t-test.

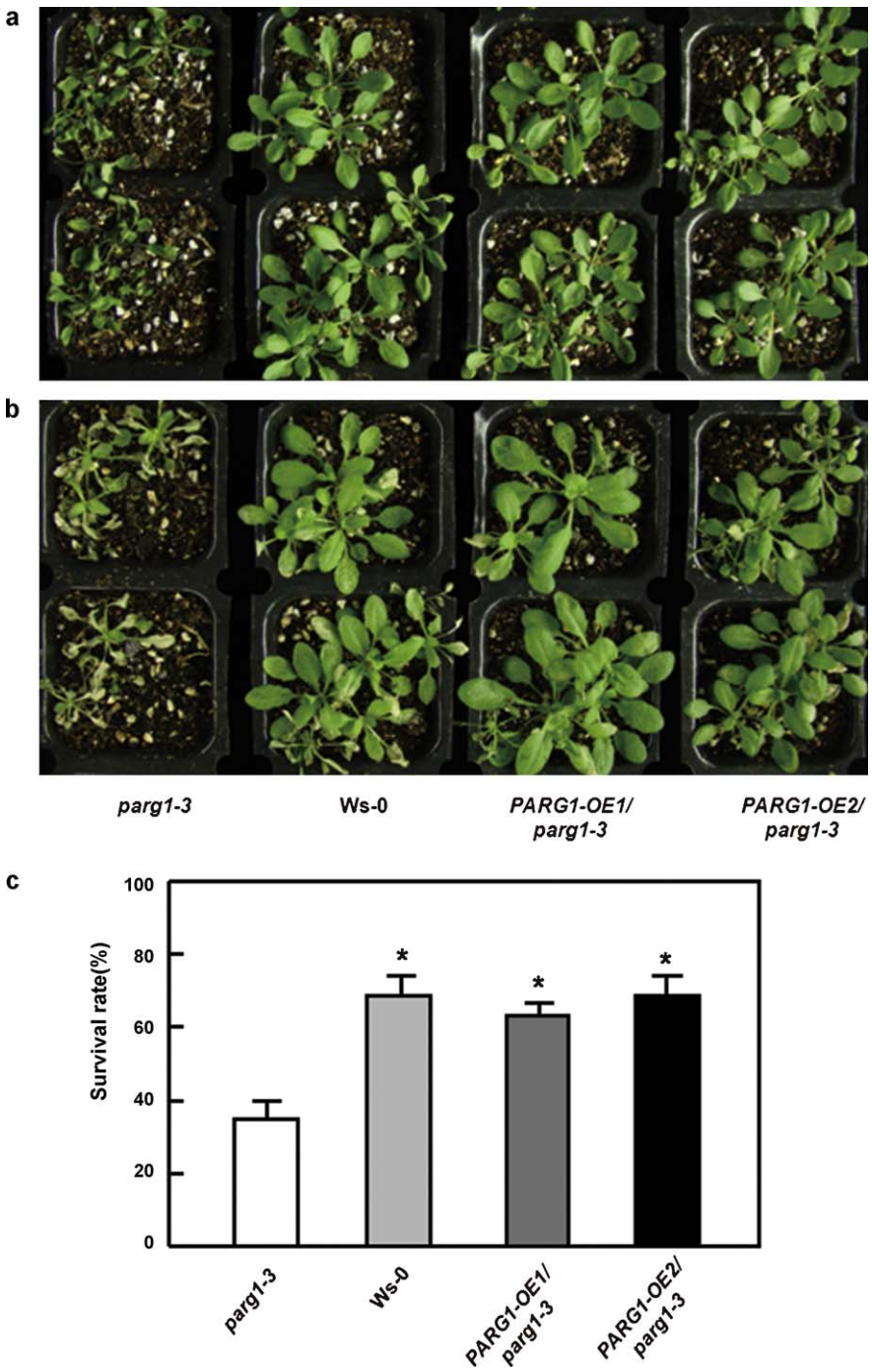

Fig. 3. Reduced drought tolerance in *parg1-3* plants. Water was withheld from three-week-old plants grown in soil. The photograph was taken on the 14th day after drought treatment (a) and on the 2nd day after re-watering (b). Survival rates of the drought stress-treated plants were determined 2 days after re-watering (c). To measure survival rates, three independent measurements, each consisting of 30 seedlings, were averaged for each plant group. Data presented are the means and standard errors from three independent experiments. Asterisks above the columns indicate significant difference at $p = 0.05$ level by *t*-test.

these findings indicate a requirement for PARG1 in osmotic stress responses in Arabidopsis.

3.3. Mutation in PARG1 depressed drought stress tolerance

We next examined and compared the phenotype of *parg1-3* mutant and transgenic overexpression lines in response to drought stress induced by withholding water from soil-grown three-week-old plants for 2 weeks. After 14 days of withholding water, almost all of the leaves of the *parg1-3* plants were completely rolled and some of the plants were wilted, whereas only a small number of the leaves of the wild type Ws-0 plants were slightly rolled (Fig. 3a). Two days after re-watering, ~70% of the wild type Ws-0 plants had recovered, but only ~34% of the *parg1-3* plants had recovered (Fig. 3b and c). After similar water withholding, four-week-old transgenic overexpression plants (*PARG1*-OE1/*parg1-3*, *PARG1*-OE2/*parg1-3*, *PARG1*-OE1/Col-0 and *PARG1*-OE2/Col-0) showed slightly more severe levels of damage than those of their corresponding wild type Ws-0 or Col-0 plants but no significant difference in survival rate was observed between the transgenic overexpression lines and the wild type controls after re-watering (Fig. 3 and supplementary Fig. 3). These

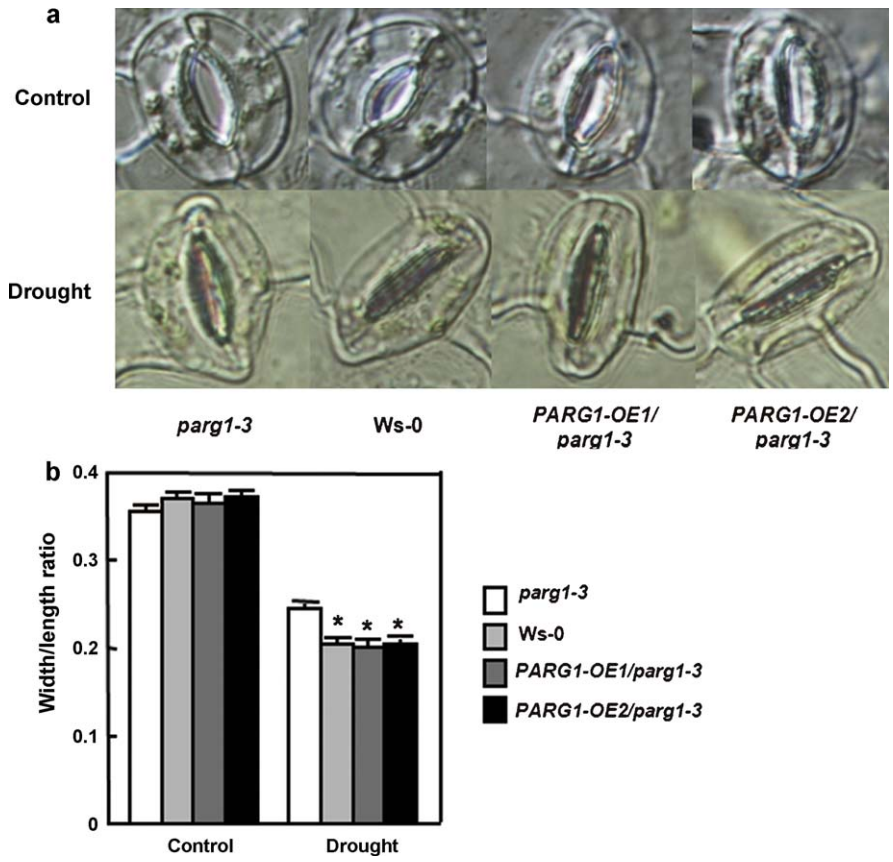

Fig. 4. Differential response of stomatal behavior to drought stress. (a) and (b) Three-week-old plants grown in soil were subjected to drought stress for 2 weeks. Photographs showing stomatal behavior were taken after treatment with drought stress (a) and at least 20 stomata on the fourth leaves were measured using a microscope (b). Data presented in (b) are the means and standard errors from three independent experiments. Asterisks above the columns indicate significant difference at $p = 0.05$ level by t-test.

results indicate that appropriate expression of *PARG1* is essential for drought stress tolerance and that excessive expression of PARG1 through overexpression approach does not further improve drought tolerance.

3.4. Mutation in PARG1 perturbed stomatal behavior under drought stress

We further examined the stomatal behavior of the *parg1-3* mutant, transgenic overexpression lines and wild type plants in response to drought stress caused by withholding water. Different stomatal behavior was observed in *parg1-3* mutant plants in response to drought stress when compared with that of the wild type Ws-0 plants. After 2 weeks of withholding water, stomata in wild type Ws-0 plants were completely closed, but stomatal apertures in *parg1-3* mutant plants remained partially open (Fig. 4a). The width/length ratios of stomatal apertures in *parg1-3* mutant plants were significantly higher than in wild type Ws-0 plants, although the ratios both in *parg1-3* mutant and wild type Ws-0 plants were markedly reduced in response to water withholding compared with the ratios in plants under normal watering conditions (Fig. 4b). These results indicate that stomatal apertures in *parg1-3* mutant plants had reduced stomatal closure in response to drought stress caused by water limitation. The stomatal responses of the transgenic overexpression lines (*PARG1*-OE1/*parg1-3*, *PARG1*-OE2/*parg1-3*, *PARG1*-OE1/Col-0 and *PARG1*-OE2/Col-0) showed similar patterns to those of the corresponding wild type Ws-0 and Col-0 plants, as revealed by similar changes of the width/length ratios in response water withholding (Fig. 4).

3.5. Mutation in PARG1 lessened oxidative stress tolerance

Because expression of *PARG1* was induced by MV treatment [33], we examined whether mutation or overexpression of *PARG1* would affect tolerance to oxidative stress during seed germination or in mature Arabidopsis leaves. In MS medium alone, without supplementation with MV, *parg1-3* mutant, transgenic overexpression line, and wild type seeds showed similar germination rates (Fig. 2a). In MS medium containing 10 μM MV, seeds from the *parg1-3* mutant line showed a lower germination rate than the wild type Ws-0 seeds (Fig. 5a). After 6 days, only 35% of the *parg1-3* mutant seeds had germinated while more than 50% of wild type seeds had germinated (Fig. 5a). Germination rates for seeds from transgenic overexpression lines (*PARG1*-OE1/*parg1-3*, *PARG1*-OE2/*parg1-3*, *PARG1*-OE1/Col-0 and *PARG1*-OE2/Col-0) in the presence of MV were comparable to those of the wild type Ws-0 and Col-0 seeds (Fig. 5a and supplementary Fig. 4a).

Exogenous application of MV by foliar spraying or by placing detached leaves in MV-containing MS solutions caused significant necrosis or bleaching, indicative of oxidative damage, on leaves from soil-grown four-week-old plants of *parg1-3* mutant, transgenic overexpression lines and the wild type Arabidopsis plants. However, oxidative damage on leaves of *parg1-3* mutant plants was much more pronounced than on wild type Ws-0 leaves (Fig. 5b and c), whereas oxidative damage on leaves of transgenic overexpression lines (*PARG1*-OE1/*parg1-3*, *PARG1*-OE2/*parg1-3*, *PARG1*-OE1/Col-0 and *PARG1*-OE2/Col-0) was comparable to that in the corresponding wild type Ws-0 and Col-0 leaves (Fig. 5b, c and supplementary Fig. 4b). MV-induced oxidative damage in leaves of *parg1-3* mutant plants reduced the chlorophyll con-

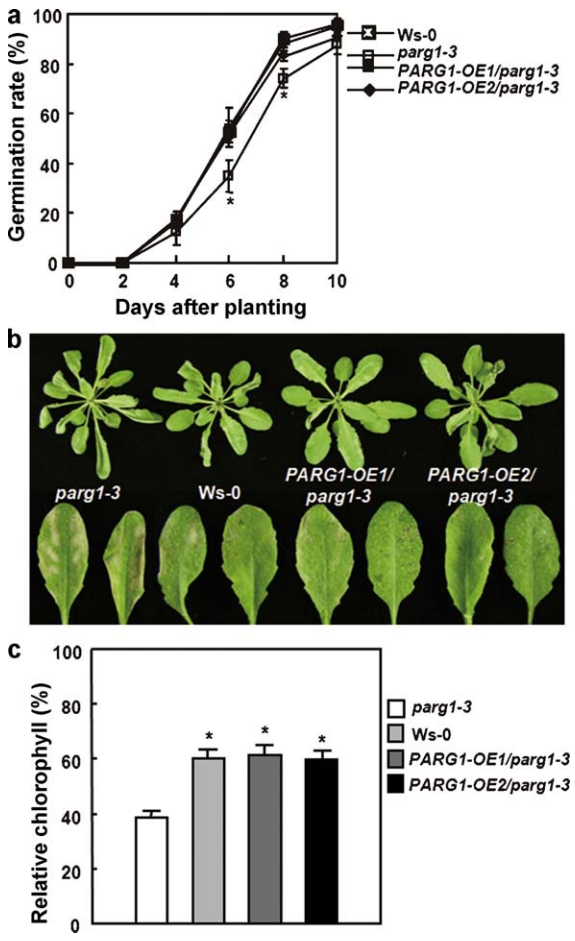

Fig. 5. Reduced tolerance to oxidative stress in *parg1-3* plants. (a) Percentages of seed germination of the *parg1-3*, *Ws-0*, *PARG1-OE1/parg1-3* and *PARG1-OE2/parg1-3* lines on 1/2 MS medium containing 10 μM MV. Seed germination was scored when the radicles emerged completely from seed coat. (b and c) Reduced oxidative stress tolerance in *parg1-3* plants. Four-week-old plants grown in soil were treated by foliar spraying with 50 μM MV. (b) Photographs showing MV-induced symptom in rosette leaves were taken 24 h or 4 d after treatments with 50 μM (upper) or 1 μM (lower) MV, respectively. (c) Chlorophyll contents in leaves were measured 4 d after treatment with 1 μM MV. Data presented in (a) and (c) are the means and standard errors from three independent experiments. Asterisks below the open squares (a) indicate significant difference at $p = 0.05$ level by *t*-test.

tent compared with that in leaves of transgenic overexpression lines and wild types. Chlorophyll content in MV-treated leaves of *parg1-3* mutant plants decreased to 40% of that in leaves without MV treatment, whereas chlorophyll contents in MV-treated leaves of transgenic overexpression lines (*PARG1*-OE1/*parg1-3*, *PARG1*-OE2/*parg1-3*, *PARG1*-OE1/Col-0 and *PARG1*-OE2/Col-0) and the corresponding wild type Ws-0 and Col-0 plants were about 60% of that in leaves without MV treatment (Fig. 5c and supplementary Fig. 4c). Mutation in *PARG1* appeared to weaken tolerance to oxidative stress, leading to an increased level of cellular damage under oxidative stress conditions. Once again, overexpression of *PARG1* did not provide further increases in oxidative stress tolerance in Arabidopsis plants.

Further confirmation of the oxidative stress tolerance phenotype in *parg1-3* plants was obtained by comparing expression of some known oxidative stress defense genes after MV treatment [3]. As shown in Fig. 6, the *parg1-3* plants showed downregulated expression of two oxidative stress defense genes, *AtAox1* (alternative oxidase 1) and *AtApx2* (ascorbate peroxidase 2), whereas expression of *AtApx1* and *AtFSD1* (Fe-superoxide dismutase 1) showed similar levels to those in the wild type plants after

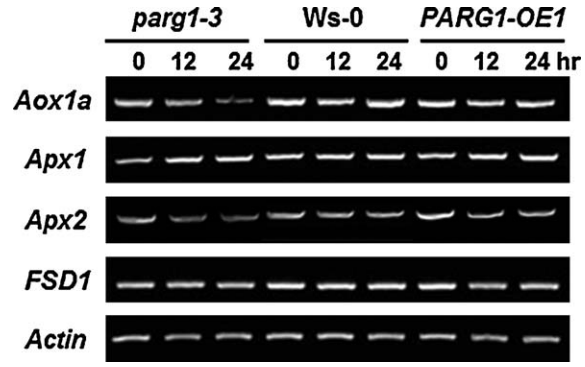

Fig. 6. Expression patterns of oxidative stress-related genes after MV treatment. Four-week-old plants were treated by foliar spraying with 25 μM MV and leaf samples were collected at different time points as indicated. Expression of oxidative stress-related genes was analyzed by RT-PCR using gene-specific primers. The actin gene was used as a control.

MV treatment. However, expression of all four oxidative stress-related genes in transgenic overexpression line *PARG1*-OE1/*parg1-3* showed similar patterns to those seen in the wild type plants after MV treatment (Fig. 6). Therefore, the reduced oxidative stress tolerance in *parg1-3* plants is likely due to a decreased capacity for anti-oxidative stress responses following MV treatment.

4. Discussion

Poly(ADP-ribosyl)ation is a unique posttranslational protein modification involved in plant responses to biotic and abiotic stresses, as shown through detailed functional analyses of PARPs and PARGs [27,28,30,31]. In the present study, use of genetic mutant and transgenic overexpression lines allowed us to examine the role of *PARG1* in tolerance to various abiotic stresses. The normal function of PARG1 is required for tolerance of Arabidopsis to drought, osmotic and MV-induced oxidative stresses, but increased expression of the *PARG1* gene does not improve abiotic stress tolerance above that seen in the wild type.

Previous studies have shown that mutations in *PARG1* and *PARG2* resulted in significant phenotypic differences in development and stress responses in Arabidopsis [31,32] indicating that PARG1 and PARG2 may function independently in Arabidopsis. In this study, we found that loss of PARG1 function by mutation led to reduced tolerance to various abiotic stresses including drought, osmotic and oxidative stress. This is supported by the observations that mutation or suppression of expression of *PARP1* and *PARP2* in Arabidopsis or oilseed rape conferred broad-spectrum stress resistance [27,28]. The balance of poly(ADP-ribose) levels, maintained by dynamic changes between the formation by PARPs and the degradation by PARGs, may therefore be critical for the activation of responses to environmental stressors in plants. In general, PARGs catalyze a reaction that degrades poly(ADP-ribose) and thus increase cellular pools of free ADP-ribose and poly(ADP-ribose), which is known as a cell death signal in mammalian cells [39]. The extent of poly(ADP-ribose) in mammalian cells is generally accepted as being directly proportional to the severity of the stress and will determine the type of cellular response, ranging from cellular defense under mild stress to cell damage under severe stress [40].

Poly(ADP-ribosyl)ation is a process that requires energy consumption [41,42]. Previous studies have demonstrated that plants with low PARP activity increased stress tolerance and that one of the mechanisms involved is due to the maintenance of energy homeostasis by reducing NAD^+ breakdown and consequent energy consumption under stress conditions [27,28]. Further analysis of the cellular level of poly(ADP-ribose) and energy homeostasis in the

parg1-3 mutant plants under normal and stress conditions could provide new evidence regarding the possible mechanism underlying PARG1 regulation of stress response in plants. Although proper function of PARG1 is clearly essential for a stress response, the absence of any phenotypic change in tolerance of seeds or adult plants of the *PARG1*-overexpressing transgenic lines to drought, osmotic or oxidative stress implies that overexpression of PARG1 confers no additional stress tolerance. However, the reason that the *PARG1* OE lines did not show any difference in drought tolerance phenotype is unclear. It is possible that the elevated mRNA in the *PARG1* OE lines may not translate into elevated levels of PARG1 protein.

Water withholding failed to induce full stomatal closure in *parg1-3* plants (Fig. 4); however, the relationship between reduced stomatal closure and decreased drought tolerance in *parg1-3* plants is still an open question. Guard cell regulation is a complex process involving several pathways that are partially or completely independent [43,44]. The possible mechanisms by which PARG1 might regulate abiotic stress responses, such as stomatal behavior, remain open questions to be investigated further. In this context, however, abscisic acid (ABA) is recognized as an essential signal molecule for the regulation of various stress responses, including stomatal closure, stress-responsive gene expression and metabolic changes [5]. A large set of ABA-responsive genes have been reported to be up-regulated in *PARP2*-silenced plants upon stress treatment, indicating that ABA signaling is likely necessary for poly(ADP-ribose)-mediated stress responses [28]. Therefore, further study is also required to clarify the possible involvement of ABA signaling in PARG1-mediated drought stress responses.

It has been reported that poly(ADP-ribosyl)ation is associated with DNA repair process [45] and oxidative stress often results in DNA damage in seed germination under normal growth conditions [46]. We thus examined the response of germination of the parg1-3 mutant seeds under oxidative stress. Our results showed that loss of PARG1 function in *parg1-3* plants resulted in reduced oxidative stress tolerance (e.g. slowed seed germination and increased leaf damage) and reduced expression of oxidative stress-related genes after MV treatment (Figs. 5 and 6). These findings are in agreement with the observation that PARP inhibitors can protect oilseed rape plants from oxidative stress [27]. Poly(ADP-ribosyl)ation has recently been reported as an important response to ROS production during pathogen defense responses and the expression of many oxidative stress-related genes was delayed or even completely abolished in *PARP2*-silenced plants [28,31]. Thus, evidence is increasing that poly(ADP-ribosyl)ation is involved in regulation of oxidative stress responses in plants. In mammalian systems, careful regulation of the cellular level of free ADP-ribose is recognized as important for minimizing the potential detrimental effects of this molecule [47,48]. In the *parg1-3* plants, excessive levels of toxic poly(ADP-ribose) may accumulate, causing cellular damage that would in turn attenuate oxidative stress tolerance. This hypothesis is partially supported by a recent study in which cellular levels of free ADP-ribose were decreased through overexpression of *NUDX2* and *NUDX7*, which code for ADP-ribose pyrophosphatases that degrade ADP-ribose. Overexpression of *NUDX2* and *NUDX7* in transgenic plants resulted in increased tolerance to oxidative stress caused by MV [33,49]. Thus, PARG1 is likely to be involved in the regulation of oxidative stress tolerance via modulation of cellular poly(ADP-ribose) reactions.

In our study, we observed that the *parg1-3* mutant seeds showed slowed rates of germination as compared with the wild type seeds under osmotic and oxidative stress conditions (Figs. 2b and 5a), although there was no significant difference in germination rates of the *parg1-3* mutant and the wild type seeds under normal condition. It should be noted that, with increasing of times in our experiments, the germination rates of the *parg1-3* mutant seeds under osmotic and oxidative stress condition showed a trend to catch up to the wild type seeds (Figs. 2b and 5a). This indicates that loss of PARG1 function in the *parg1-3* mutant may affect the germination processes of the seeds. This is similar to the observation that mutation in DNA ligase 6 resulted in a slowing of the germination processes under cold stress [50]. Because poly(ADP-ribosyl)ation is associated with DNA repair process [49], the slowed germination rates of the *parg1-3* mutant seeds under stress conditions thus may suggest that poly(ADP-ribosyl)ation-associated DNA damage and repair pathways are important to seed germination.

In conclusion, we present genetic evidence in support of a role for PARG1 in abiotic stress tolerance in plants which probably involves maintaining the cellular balance of poly(ADP-ribose). However, further investigation is required to clarify many open questions regarding the physiological and biochemical mechanisms underlying PARG activity (e.g., the mode of action and the targets of PARG1 in plant cells) that synergistically regulate abiotic stress responses in plants.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.plantsci.2010.09.002.

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