Plant Cell Reports (2018) 37:453–465
https://doi.org/10.1007/s00299-017-2242-9

ORIGINAL ARTICLE

Enhanced multiple stress tolerance in *Arabidopsis* by overexpression of the polar moss peptidyl prolyl isomerase *FKBP12* gene

Hemasundar Alavilli[1] · Hyoungseok Lee[2] · Mira Park[1,2] · Dae-Jin Yun[3] · Byeong-ha Lee[1]

Received: 29 October 2017 / Accepted: 5 December 2017 / Published online: 15 December 2017

Abstract

Key message ***PaFKBP12*** **overexpression in** ***Arabidopsis*** **resulted in stress tolerance to heat, ABA, drought, and salt stress, in addition to growth promotion under normal conditions.**

Abstract *Polytrichastrum alpinum* (alpine haircap moss) is one of polar organisms that can withstand the severe conditions of the Antarctic. In this study, we report the isolation of a peptidyl prolyl isomerase *FKBP12* gene (PaFKBP12) from *P. alpinum* collected in the Antarctic and its functional implications in development and stress responses in plants. In *P. alpinum, PaFKBP12* expression was induced by heat and ABA. Overexpression of *PaFKBP12* in *Arabidopsis* increased the plant size, which appeared to result from increased rates of cell cycle. Under heat stress conditions, *PaFKBP12*-overexpressing lines (*PaFKBP12*-OE) showed better growth and survival than the wild type. *PaFKBP12*-OE also showed higher root elongation rates, better shoot growth and enhanced survival at higher concentrations of ABA in comparison to the wild type. In addition, *PaFKBP12*-OE were more tolerant to drought and salt stress than the wild type. All these phenotypes were accompanied with higher induction of the stress responsive genes in *PaFKBP12*-OE than in the wild type. Taken together, our findings revealed important functions of *PaFKBP12* in plant development and abiotic stress responses.

Keywords FK506 binding protein · FKBP12 · Peptidyl prolyl isomerase · Stress tolerance · *Arabidopsis thaliana* · *Polytrichastrum alpinum*

Introduction

Mosses are the dominant flora in Antarctic landscape vegetation (Turetsky et al. 2012). During their life cycle, mosses encounter several major stresses such as drought, extreme temperatures, and salinity that cause irreversible damages to plant survival. However, Antarctic mosses evolved with diverse adaptive strategies to survive and overcome the adverse conditions. Thus, it is presumable that Antarctic mosses would contain the genetic resources for crop improvement against environmental stress. *Polytrichastrum alpinum*, also known as alpine haircap moss, grows over the Antarctic as well as humid rocky substrates, sub-arctic, and montane ice-free areas (Zuniga-Gonzalez et al. 2016). Its survival under severe conditions implicates the presence of useful genes for stress tolerance in the *P. alpinum* genome.

FK506-binding proteins (FKBPs) belong to the peptidyl prolyl *cis–trans* isomerase (PPIases, EC 5.1.2.8) superfamily that catalyzes the *cis* to *trans* conformation of the N-terminal peptide bond of proline residues in peptide or protein substrates (Fischer et al. 1989). Originally, FKBPs were identified as a receptor for immunosuppressant drugs such as FK506 and rapamycin in mammalians and is now found to exist in Archaebacteria to plants and mammals (Breiman and Camus 2002; Harding et al. 1989; Siekierka et al. 1989). Structurally, FKBPs are characterized by the conserved FK506-binding (FKB) domain that is important for FK506 binding and PPIase activities (Barik 2006;

Communicated by Youn-Il Park.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00299-017-2242-9) contains supplementary material, which is available to authorized users.

✉ Byeong-ha Lee
byeongha@sogang.ac.kr

[1] Department of Life Science, Sogang University, Seoul 04107, South Korea

[2] Unit of Polar Genomics, Korea Polar Research Institute, Incheon 21990, South Korea

[3] Department of Biomedical Science and Engineering, Konkuk University, Seoul 05029, South Korea

Fanghanel and Fischer 2004). FKBP members contain one to three FKB domains (Galat 2003). While small size FKBP members consists of one FKB domain only, big size FKBPs contain up to three FKB domains along with protein–protein interacting domains such as tetratricopeptide repeat domains (TPR) and calmodulin-binding domains (CaM-BDs) (Galat 2003; He et al. 2004; Rulten et al. 2006). In addition to the structural variations, FKBPs are diversely localized in subcellular compartments including cytoplasm, nucleus, mitochondria, chloroplast, endoplasmic reticulum (Barik 2006; Gollan et al. 2012), suggesting the diverse functions of FKBPs. FKBPs are involved in many important biological processes including T-cell activation, cell proliferation, protein folding and transcriptional regulation in immune responses (Bonner and Boulianne 2017; Nath and Isakov 2015). Plant FKBPs have also been implicated in development, stress response, transcription regulation and chloroplast function (Gollan et al. 2012). For example, *Arabidopsis* FKBP62 and FKBP65 (as known as ROF1 and ROF2, respectively) functions in high temperature stress tolerance by regulating small heat shock proteins (Meiri and Breiman 2009; Meiri et al. 2010). In addition, the *Arabidopsis* mutants defective in FKBP72/PASTICCINO1 or FKBP42/TWISTED DWARF1 displayed developmental defects which resulted from alterations of FKBP27-mediated cytokinin action or FKBP42-activated auxin transport (Bailly et al. 2006; Bouchard et al. 2006; Faure et al. 1998; Vittorioso et al. 1998).

Compared to other organisms, plants contain higher numbers of FKBP paralogs in their genome (Geisler and Bailly 2007; Gollan et al. 2012). For instance, there are 23 *FKBP* genes in *Arabidopsis thaliana* (He et al. 2004), 23 in *Chlamydomonas reinhardtii* (Ahn et al. 2010), 29 in *Oryza sativa* (Gollan and Bhave 2010), 30 in *Zea mays* (Wang et al. 2012) while it was only 3 in *Escherichia coli* (Romano et al. 2004), 4 in *Saccharomyces cerevisiae* (He et al. 2004; Romano et al. 2004), 8 in *Caenorhabditis elegans* (He et al. 2004), 7 in *Drosophila melanogaster* (Ahn et al. 2010) and 15 in *Homo sapiens* (Liu et al. 2017). Therefore, high numbers of plant FKBP genes suggest the functional diversity in their physiological functions distinct from other organisms' FKBPs.

FKBP12 is a smallest FKBP that is localized in cytoplasm (Gollan et al. 2012). Several studies established the *FKBP12* role as an intracellular receptor for immunosuppressant FK506 or rapamycin that mediates immunosuppression of T lymphocyte-cell activation in mammals and antimicrobial actions in fungi (Arévalo-Rodríguez et al. 2004). FK506/rapamycin-bound FKBP12 inhibits the phosphatase activity of calcineurin and/or the kinase activity of Target of rapamycin (TOR) resulting in T-cell inactivation (Breiman and Camus 2002; Choi et al. 1996; Geisler and Bailly 2007; Sabatini et al. 1994). Even in the absence of FK506 or rapamycin, FKBP12 can also regulate the activities of calcium channels, such as the ryanodine receptor (RyR) and the inositol (1,4,5)-triphosphate receptor, by direct interaction (Breiman and Camus 2002; Geisler and Bailly 2007). In plants, FKBP12 was reported to have its roles in abiotic stress responses, growth and development. For example, the overexpression of the thermophilic microalga *Scenedesmus FKBP12* (*Sce.FKBP12*) in *E. coli* resulted in heat and salt stress tolerance (Subin et al. 2016). *Picea wilsonii FKBP12* (PwFKBP12) interacts with *HAPLESS5* transcription factor to regulate the pollen tube growth orientation (Yu et al. 2011). Although plant FKBP12s share high sequence similarity with those of yeast and mammal FKBP12 proteins, FKBP12 proteins from many plants including *Arabidopsis* do not seem to bind to FK506 or rapamycin to form FK506–FKBP12 complex to inhibit TOR complex (Menand et al. 2002), while maize or Chlamydomonas FKBP12 bind to FKB506 or rapamycin (Agredano-Moreno et al. 2007; Crespo et al. 2005), implicating the evolutionary diversity of FKBP12s among the plant species. Thus, it now becomes clear that FKBP12 are diverse among the plants species and plays crucial roles in plants. To understand the functions of FKBP12, we isolated a gene encoding a stress responsive FKBP12 from the Antarctic moss *Polytrichastrum alpinum* (*PaFKBP12*) and found the overexpression of *PaFKBP12* resulted in enhanced tolerance to multiple stresses including high temperature, ABA, drought and salt stress.

Materials and methods

Conserved domain analysis and phylogenetic analysis

Sequences of FKBP12 protein homologs from different species were collected from NCBI Blast suite using the *PaFKBP12* protein sequence. Multiple sequence alignments were performed using ClustalW (Thompson et al. 1994). Conserved domains in each sequence were identified using NCBI conserved domain finder (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Alpha helix (α) and beta sheet (β) regions of all the FKBP12 variants were labelled according to the *Homo sapiens* FKBP12 tertiary structure (PDB ID, 2PPN) (Szep et al. 2009). Phylogenetic tree was constructed using maximum likelihood method in molecular evolutionary genetic analysis (MEGA) software version 6 (Tamura et al. 2013).

Plant material and growth conditions

Polytrichastrum alpinum (Hedw.) S.L.Sm. samples were obtained from the King Sejong Antarctic station (62°14 × 29″S; 58°44 × 18″W) at the Barton Peninsula of King George Island. Moss gametophores was cultured

on BCD solid media (Ashton and Cove 1977), frequently used for *Physcomitrella patens* culture (Du et al. 2016) at 15 °C with a 16 h light/8 h dark cycle and a light intensity of 150 µmol m^{-2} S^{-1}.

Arabidopsis thaliana (accession Columbia 0) seeds were planted onto a petri dish containing Murashige Skoog (MS) medium (JRH Biosciences, USA) supplemented with 2% sucrose and 0.3% gelrite. After 2 day stratification at 4 °C, the plates were placed at 22 ± 1 °C and a relative humidity of 70% under continuous light (80–100 µmol m^{-2} s^{-1}) as previously described (Alavilli et al. 2017). When necessary, seedlings raised on MS plates were transferred to soil pots (Sun Gro propagation mixture, Canada) and grown in a growth chamber at 22 ± 1 °C and 50–70% relative humidity with a 16 h light/8 h dark cycle.

Cloning of *PaFKBP12* gene and generation of transgenic *Arabidopsis*

Total RNA was isolated from *P. alpinum* using the RNeasy plant mini kit (QIAGEN, Germany) and first strand cDNA was prepared with MMLV reverse transcriptase (Enzynomics, Korea) using the gene specific primers (Suppl. Table 1). The resultant PCR products were cloned into pENTR/D/TOPO vector (Invitrogen, USA), and the sequence was verified using M-13 primers. After confirmation, the entry plasmids were LR-recombined with the binary destination vector, pMDC32 (Curtis and Grossniklaus 2003) and the resultant construct was named pMDC32-35S:*PaFKBP12*, which was then transferred into *Agrobacterium tumefaciens* strain GV3101 via electroporation. Agrobacterium-mediated transformation of *Arabidopsis thaliana* (Col-0) was performed through floral dipping (Clough and Bent 1998). For selection of *PaFKBP12*-OE lines, T1 seeds were harvested from the floral dipped plants and selected on plates containing hygromycin (25 µg/ml). The transgenic selections were continued until T4 generation to obtain homozygotes for the transgene. The two lines (*PaFKBP12*-OE1 and *PaFKBP12*-OE2) were selected for further phenotypic characterization. Hygromycin resistance of the T2 generation from each line was segregated in 3:1 ratio of resistant to sensitive, suggesting a single locus of insertion.

Stress treatment and phenotype analysis

Polytrichastrum alpinum gametophores cultured at 15 °C were transferred onto fresh agar plates of BCD medium containing mannitol (300 mM), NaCl (150 mM) or ABA (100 µM) and incubated for designated hours. Heat treatment was carried out by transferring colonies grown at 15 °C to chamber operating at 37 or 42 °C for 2 h. For stress-responsive gene expression analysis, 12–14–day-old Arabidopsis plants were treated with dehydration (placed on a filter paper in room temperature) for 3 h or heat (45 °C) for 3 h or ABA (100 µM) for 3 h.

For *Arabidopsis thaliana* germination experiments, at least 100–120 seeds of each genotype were planted in a media with or without stress agent and the germination rates were scored on the third day after transfer from stratification conditions (42 °C, 2 days) to normal conditions (22 °C). The germination rates were expressed as a percentage of germinated seeds in a total number of seeds plated. For root growth experiments, the seeds were planted in vertical MS plates supplemented with 0.6% gelrite and allowed to grow for 4 days. Seedlings with 1–1.5 cm long root were transferred onto a second MS vertical plate supplemented with different concentrations of stress agent as previously described (Alavilli et al. 2017). For a better comparison of root length, relative values (root lengths of each line to ones their respective controls growing on normal MS medium) were used. The hypocotyl elongation under heat stress was assessed as described previously (Hong and Vierling 2000). The chlorophyll content in control and stress-treated plants were determined by the methods previously described (Lichtenthaler 1987). For water loss analysis, rosette leaves of 3 weeks old seedlings were detached, weighed and placed on paper to impose drought stress. Fresh weights of rosette leaves were measured at 0, 1, 2, 4 and 6 h. Water loss was expressed as a loss in fresh weight as described previously (Alavilli et al. 2016). For drought stress analysis in the soil grown plants, 16-day-old seedlings were subjected to desiccation by withholding water supply for 32 days and then re-watered. Survival percentages were recorded 4 days after the recovery.

Stomatal aperture assay

Stomatal aperture was measured as previously described by (Ha et al. 2016). Briefly, epidermal peels from rosette leaves of 3–4-week-old *Arabidopsis* plants were floated in the stomatal opening solution (SOS; 10 mM KCl, 50 µM $CaCl_2$, 10 mM MES, pH 6.15) and exposed to light for 2 h to achieve full opening of stomata. Subsequently, ABA was added to the SOS to the final concentration of 0 or 10 µM and incubated for 2 more hours to induce stomatal closing. After incubation, stomatal apertures were photographed and stomatal apertures of the seedlings were measured (width/length) in the presence or absence of 10 µM ABA (at least 20 stomatal apertures were measured for each) using the Image J.

Gene expression analysis

Total RNAs were isolated from plant materials using RNAiso Plus reagent (Takara Bio, USA) followed by DNase I treatment (New England Biolabs, USA) to eliminate

genomic DNA contamination. cDNAs was synthesized using the TOP script reverse transcriptase kit (Enzynomics, Korea) according to the manufacturer's instructions. The quantitative RT-PCR was performed using KAPA SYBR FAST qPCR kit (Kapa Biosystems, USA) with the Light cycler 96 system (Roche, Germany). The relative expression of *PaFKBP12* in *P. alpinum* was calculated by normalizing the expression values with that of housekeeping *PaTubulin* gene. For the *Arabidopsis*, relative gene expression analysis was carried out using *AtClathrin* as an internal control. The ΔΔ Ct method (Livak and Schmittgen 2001) was adopted to calculate the relative gene expression. Primer pairs used for amplifications are shown in Suppl. Table 1.

Propidium iodide staining

For cell size comparison, leaves of 4–5 days old soil grown *Arabidopsis* seedlings were collected and dipped in propidium iodide (1 mg/ml stock) solution for 1–2 min. Seedlings were quickly rinsed in water before mounting them on to a glass and then observed under confocal microscope (Leica, Germany) using excitation maxima at 546 nm.

Statistical analysis

All statistical comparisons between the variances were determined by ANOVA (Analysis of variance) and least significant differences (LSD) between the variants were calculated using Statistix 8.1 computation software. Statistically significant mean values were denoted as * (P value ≤ 0.05).

Results

Sequence alignment and phylogenetic analysis of *PaFKBP12*

The coding sequence of *PaFKBP12* (339 nucleotides) was retrieved from our unpublished transcriptome data, based on its sequence homology with *Arabidopsis thaliana FKBP12* (*AtFKBP12*). The PaFKBP12 coding sequence was 339 nucleotides in length with an 112 deduced amino acid sequence. The computational analysis on the PaFKBP12 protein estimated a molecular mass of 12.1 kDa and an isoelectric point of 8.62 (http://web.expasy.org/cgi-bin/compute_pi/pi_tool). Homologous *FKBP12* gene sequences from other species were obtained by BLAST search to study the evolutionary relationships of PaFKBP12 with the ones in other species. Multiple sequence alignment of PaFKBP12 protein sequence revealed that it shared a 73–83% identity with its plant homologs, while a slightly low identity (44–50%) was observed when compared with its animal homologs (Fig. 1). PaFKBP12 was expected to contain only one FKB conservative domain (Fig. 1a).

It is known that FKBP12 activity as peptidyl prolyl isomerase is dependent on the 13 conserved amino acid residues that are important for its binding to immunosuppressant FK506 or rapamycin (DeCenzo et al. 1996).

In our sequence alignments, these 13 conserved residues remained unchanged in the animal homologs, whereas the plant FKBP12 orthologues had the 9 amino acid residues conserved and the 4 amino acids different from the animal residues. However, each substituted amino acid of these 4 amino acids were the same at each residue of all plant FKBP12 homologs indicating plant-specific differentiation of the FKBP12 gene (Fig. 1a). Phylogenetic analysis also revealed a clear divergence of FKBP12 proteins between animal and plant FKBP12 proteins (Fig. 1b). Even within the plant FKBP12 clade, all the non-vascular plant FKBP12 orthologues were diverged away from vascular plants and grouped together, reflecting well the evolutionary modifications of FKBP12 within the plant species (Fig. 1b).

Expression of *PaFKBP12* in *P. alpinum* under various stress conditions

We examined the *PaFKBP12* transcript abundance under temperature (heat and cold), ABA, osmotic, and salt stress conditions in *P. alpinum*. For this, quantitative real time PCR (qRT-PCR) analysis was carried out with total RNA isolated from *P. alpinum* gametophores treated with high temperatures (37 and 42 °C for 2 h), ABA (100 µM for 1 and 3 h), mannitol (300 mM for 6 and 72 h), NaCl (150 mM for 6 and 72 h) or for cold (4 °C for 6 or 24 h). The results revealed that *PaFKBP12* transcript levels were increased in response to both heat and ABA treatments, but not to other stress treatments (Fig. 2). Rather, PaFKBP12 was down-regulated at the early time point after 150 mM NaCl treatment. Thus, we concluded that *PaFKBP12* is a heat and ABA-inducible gene in *P. alpinum*.

Generation and morphology of *PaFKBP12* overexpressing lines

To examine the functional roles of *PaFKBP12* in plants, we generated transgenic *Arabidopsis* lines overexpressing *PaFKBP12* gene under the control of cauliflower mosaic virus (CaMV) 35S promoter (35S:*PaFKBP12*). Using hygromycin resistance selection and the presence of the 35S:*PaFKBP12* transgene, we selected stable homozygous transgenic lines for 35S:*PaFKBP12* in T4 generation (hereafter referred to as *PaFKBP12*-OE lines, where OE stands for overexpressing). The semi-quantitative RT-PCR results demonstrated that the transgenic lines were overexpressing

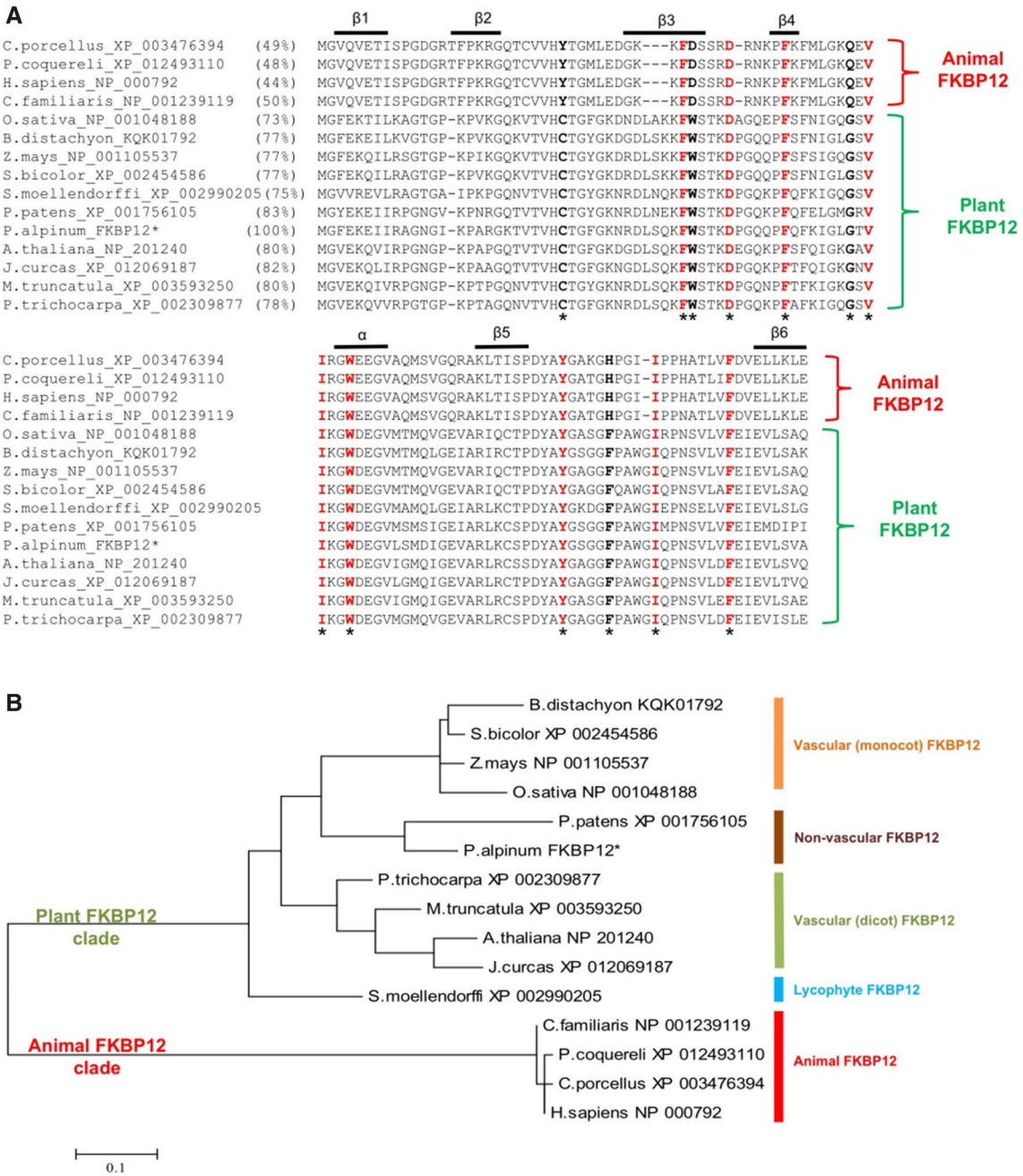

Fig. 1 Alignment and phylogenetic analysis of *PaFKBP12* homologs. **a** Sequences aligned are from the FKBP homologs of *Polytrichastrum alpinum, Physcomitrella patens* (XP_001756105), *Jatropha curcas* (XP_012069187), *Medicago truncatula* (XP_003593250), *Arabidopsis thaliana* (NP_201240), *Populus trichocarpa* (XP_002309877), *Zea mays* (NP_001105537), *Brachypodium distachyon* (KQK01792), *Sorghum bicolor* (XP_002454586), *Oryza sativa* (NP_001048188), *Selaginella moellendorfi* (XP_002990205), *Homo sapiens* (NP_000792), *Propithecus coquereli* (XP_012493110), *Canis familiaris* (NP_001239119), *Cavia porcellus* (XP_003476394). Bars span alpha helix (α) and beta sheet (β) regions drawn based on tertiary structure of *H. sapiens* FKBP12 (PDB ID, 2PPN). Asterisks below the alignments indicate the amino acid residues crucial for PPIase activity of *Homo sapiens* FKBP12. The numbers in parentheses indicate the identity values of each homolog in comparison with PaFKBP12. **b** Phylogenetic tree of FKBP12 proteins from 15 different species. The phylogenetic tree was constructed using maximum likelihood method in MEGA 6 software program

the *PaFKBP12* gene (Fig. 3a). We used the two independent lines (*PaFKBP12*-OE1 and *PaFKBP12*-OE2) for further phenotypic analyses and abiotic stress response evaluation.

Three-week-old *PaFKBP12*-OE plants grown under normal conditions displayed enlarged shoots than the WT plants (Fig. 3b). The fresh weight measurement of the

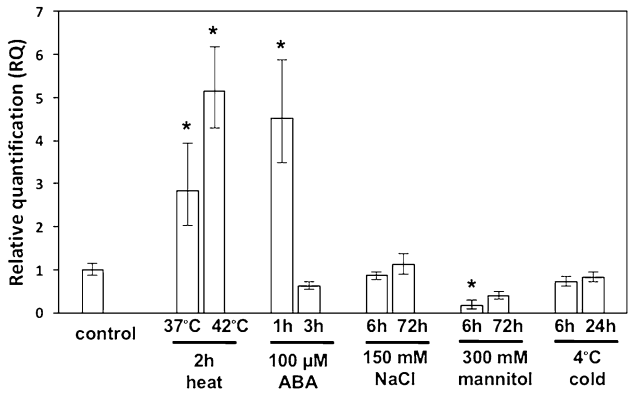

Fig. 2 Expression of *PaFKBP12* in *P. alpinum* under various stress conditions. The *PaFKBP12* expression levels were measured by quantitative PCR with total RNA from *P. alpinum* gametophores under heat stress (37 or 42 °C for 2 h), 100 µM ABA (1 or 3 h), 300 mM mannitol (6 or 72 h), 150 mM NaCl (6 or 72 h), and cold (4 °C for 6 or 24 h). The *P. alpinum tubulin (PaTubulin)* gene was used as an internal control for normalization. The expression level of *PaFKBP12* at 0 h was used as a calibrator for quantification and was assumed as 1. Error bars represent standard deviation of mean of three biological repeats. Asterisks indicate statistical significance in LSD test ($P < 0.05$)

seedlings confirmed the enhanced growth of *PaFKBP12*-OE lines. Fresh weights of the 3-week-old transgenic plants ranged between 101 and 106 mg/seedling while those of WT were 72 mg/seedling (Fig. 3c). To examine the reasons of enhanced growth in *PaFKBP12*-OE, we measured the cell sizes in the leaves of WT and *PaFKBP12*-OE lines. Compared to the WT, the *PaFKBP12*-OE lines showed smaller cell size in both adaxial and abaxial surfaces of the leaves, suggesting that the enlarged leaves might be because of enhanced cell cycle rate in the *PaFKBP12*-OE lines (Fig. 3d). We also noticed that overexpression of *PaFKBP12* largely increased the seed number per silique in the transgenic lines (Fig. 3e, f).

Heat stress tolerance in *PaFKBP12* overexpressing lines

As we observed the heat-inducibility of *PaFKBP12* in *P. alpinum*, we examined the roles of *PaFKBP12* under heat stress in the *PaFKBP12*-OE lines. Four to five days old vertically grown seedlings were heat-treated at 45 °C for 1 h and allowed to recover for 8 days. After the heat treatment, the survival of seedlings and the root length were measured as

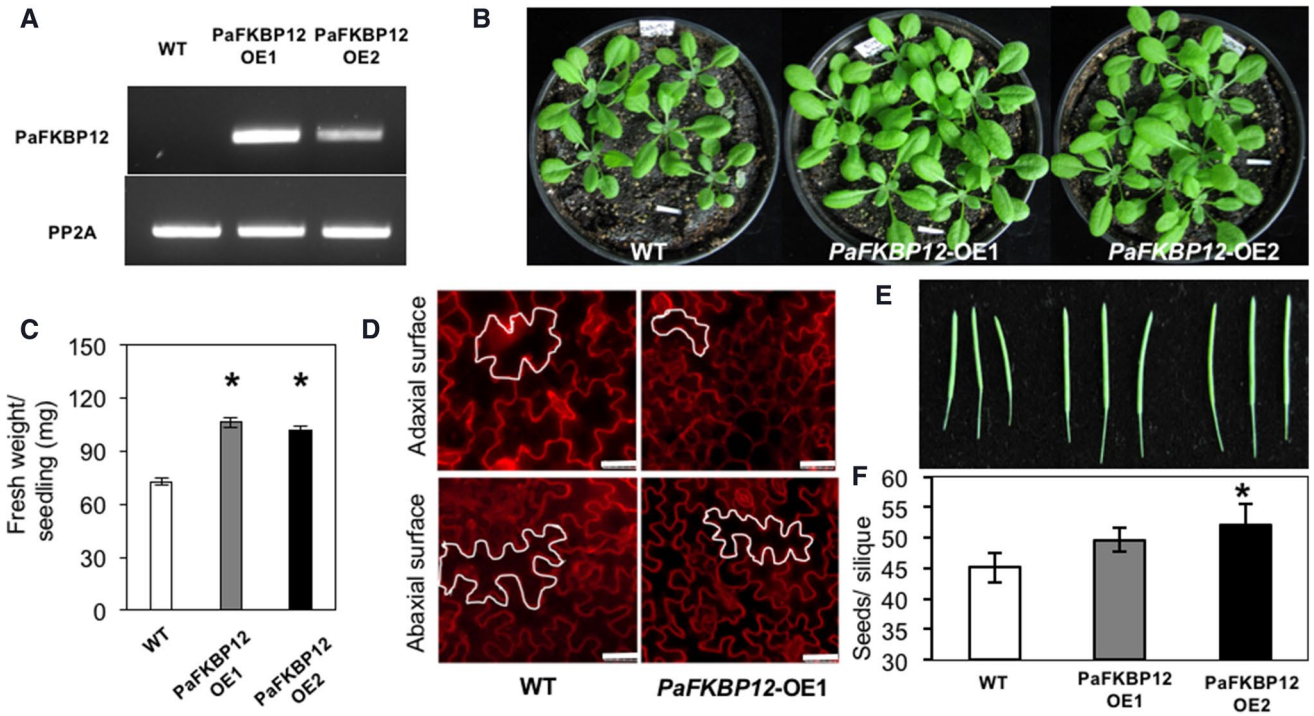

Fig. 3 Growth and development of *PaFKBP12*-OE lines. **a** Confirmation of transgene overexpression in *Arabidopsis* transgenic plants by semi-quantitative RT-PCR. *AtPP2A* was used as internal control. **b** Size comparison between WT and *PaFKBP12*-OE at the vegetative stage (3 week after germination). **c** Measurements of fresh weight per seedling of WT and *PaFKBP12*-OE ($n = 20$). **d** Leaf epidermal cell size comparison between WT and *PaFKBP12*-OE seedlings (4–5 day old) grown under normal growth conditions. Propidium iodide stained leaves were observed under confocal microscope. **e** Silique size comparison between WT and *PaFKBP12*-OE. **f** Comparison of seed number per silique between WT and *PaFKBP12*-OE ($n = 10$). Error bars represent standard deviation and asterisks indicate statistical significance in LSD test ($P < 0.05$)

heat stress tolerance indexes. Most of *PaFKBP12*-OE seedlings survived the heat treatment while most of WT seedlings died after the treatment (Fig. 4a, b). Moreover, the root elongation rate was higher in PaFKBP12-OE than in WT (26.60–26.63% relative root growth in *PaFKBP12*-OE vs. 3.87% in WT) (Fig. 4c).

We also carried out hypocotyl elongation assay which was previously described as one of heat tolerance phenotype analyses (Hong and Vierling 2000). We observed no notable differences in etiolated hypocotyl growth between *PaFKBP12*-OE and WT plants after 2.5 days of growth. However, when exposed to 45 °C for 2 h, *PaFKBP12*-OE lines were able to produce longer hypocotyls compared to WT (Fig. 4d, e), indicating the enhanced basal heat tolerance in *PaFKBP12*-OE. Similarly, we also examined acquired heat tolerance by pre-treating seedlings at 38 °C for 90 min before the 45 °C treatment. The *PaFKBP12*-OE lines still had longer hypocotyls than the WT plants did. Taken together with the previous basal heat tolerance test, these results suggest that *PaFKBP12*-OE lines are less sensitive to both basal and acquired heat stresses than the WT plants, and *PaFKBP12* promotes thermotolerance via both basal and acquired thermotolerance modes (Fig. 4d, e).

ABA hyposensitivity in *PaFKBP12* overexpressing lines

Abscisic acid regulates several key processes such as seed germination, plant development and various abiotic stress responses (Kim 2014). The expression of *PaFKBP12* gene was dramatically induced in *P. alpinum* by ABA treatment (Fig. 2). To understand the roles of *PaFKBP12* in response to ABA, we investigated the growth of *PaFKBP12*-OE at both germination and post-germination stages under ABA treatment. For germination analysis, the seeds of WT and *PaFKBP12*-OE were planted on MS plates supplemented

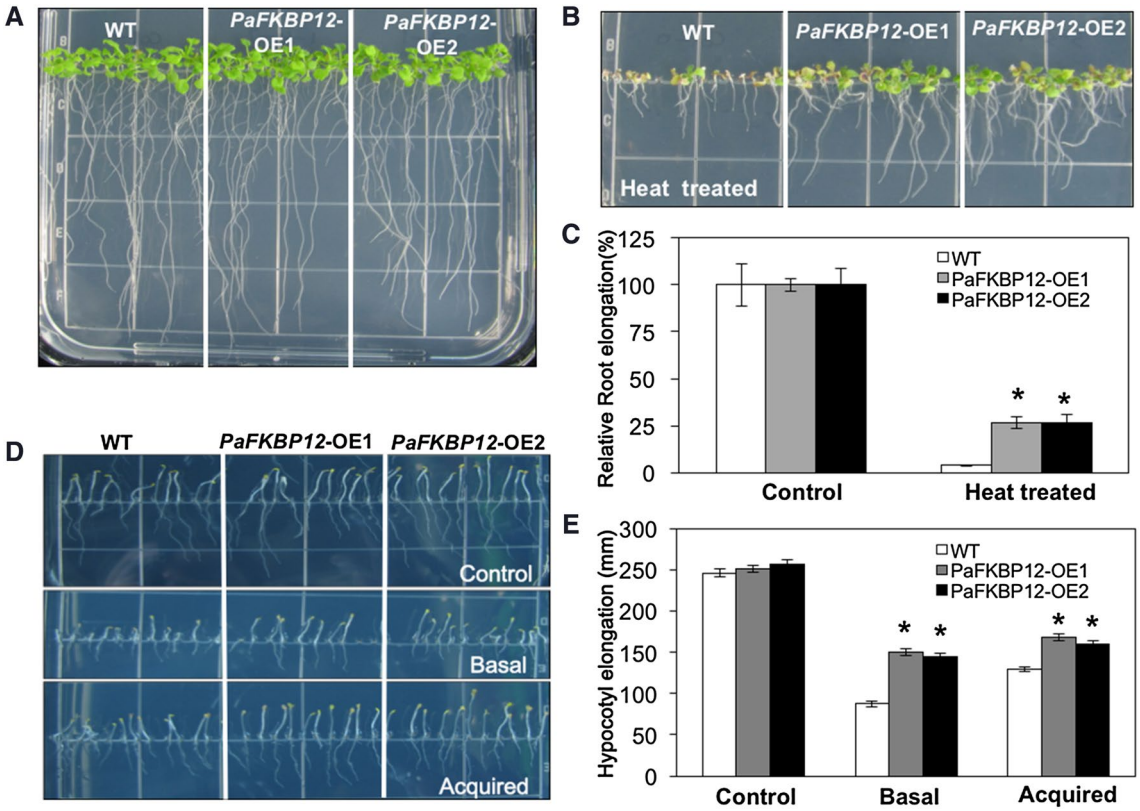

Fig. 4 Heat stress tolerance in *PaFKBP12*-OE lines. **a, b** Three to four days old seedlings of WT and *PaFKBP12*-OE vertically grown on MS plates were either untreated (**a**) treated (**b**) with 45 °C for 1 h and recovered at 22 °C for 8 days before the pictures were taken. **c** Quantitative analysis of root elongation of WT and *PaFKBP12*-OE lines. Root lengths were measured after 8 days of heat stress recovery. **d** Hypocotyl elongation analysis of WT and *PaFKBP12*-OE after heat stress. After growth for 2.5 days in the dark at 22 °C, seedlings underwent the continuous growth at 22 °C (control), heat treatment at 45 °C for 2 h (basal), or heat treatment at 45 °C for 2 h after a pre-treatment of 38 °C for 90 min and 22 °C 2 h (acquired). After the treatments seedlings were returned to 22 °C and allowed to recover for 2.5 days before seedling hypocotyl elongations were photographed and measured. **e** Quantitative analysis of the hypocotyl elongation of WT and *PaFKBP12*-OE lines. Error bars represent standard deviation of the mean values of three independent experiments. Asterisks indicate statistical significance in LSD test ($P < 0.05$)

with different concentrations of ABA. Three days after planting, the germination rates of each genotype were compared and no differences between WT and *PaFKBP12-OE* were observed (Fig. 5a).

Next, we compared root growth patterns of WT and *PaFKBP12*-OE under different ABA concentrations. Three to four days old seedlings grown under normal conditions were transferred on to the MS plates containing different ABA concentrations. After 8 days of transfer, root lengths of all the lines were similar in the control MS media. However, the relative root elongation in *PaFKBP12*-OE lines were significantly higher than that of WT in the presence of 2.5 µM ABA (81.1–84.9 vs. 66.5%), 5 µM ABA (73.1–74 vs. 56.1%) or 10 µM ABA (70–72.3 vs. 54.2%) (Fig. 5b, c). Fresh weights of the *PaFKBP12*-OE seedlings grown on ABA medium was also examined. For this, seedlings grown under normal conditions were transferred to MS medium containing 5 µM ABA and measured the fresh weight after 8 days of transfer. Consistent with the longer root in *PaFKBP12*-OE under ABA treatment, relative fresh weight of *PaFKBP12*-OE lines was significantly higher than WT in presence of 5 µM ABA (69–70 vs. 51.1%) (Fig. 5d). We also examined the seedling responses under prolonged ABA treatment. Prolonged stress under 1 µM ABA caused chlorophyll bleaching in WT, but not in *PaFKBP12*-OE lines (Fig. 5e, f). Collectively, these results suggest that *PaFKBP12* overexpression reduces the sensitivity to ABA in post-germination stage but not at the germination stage.

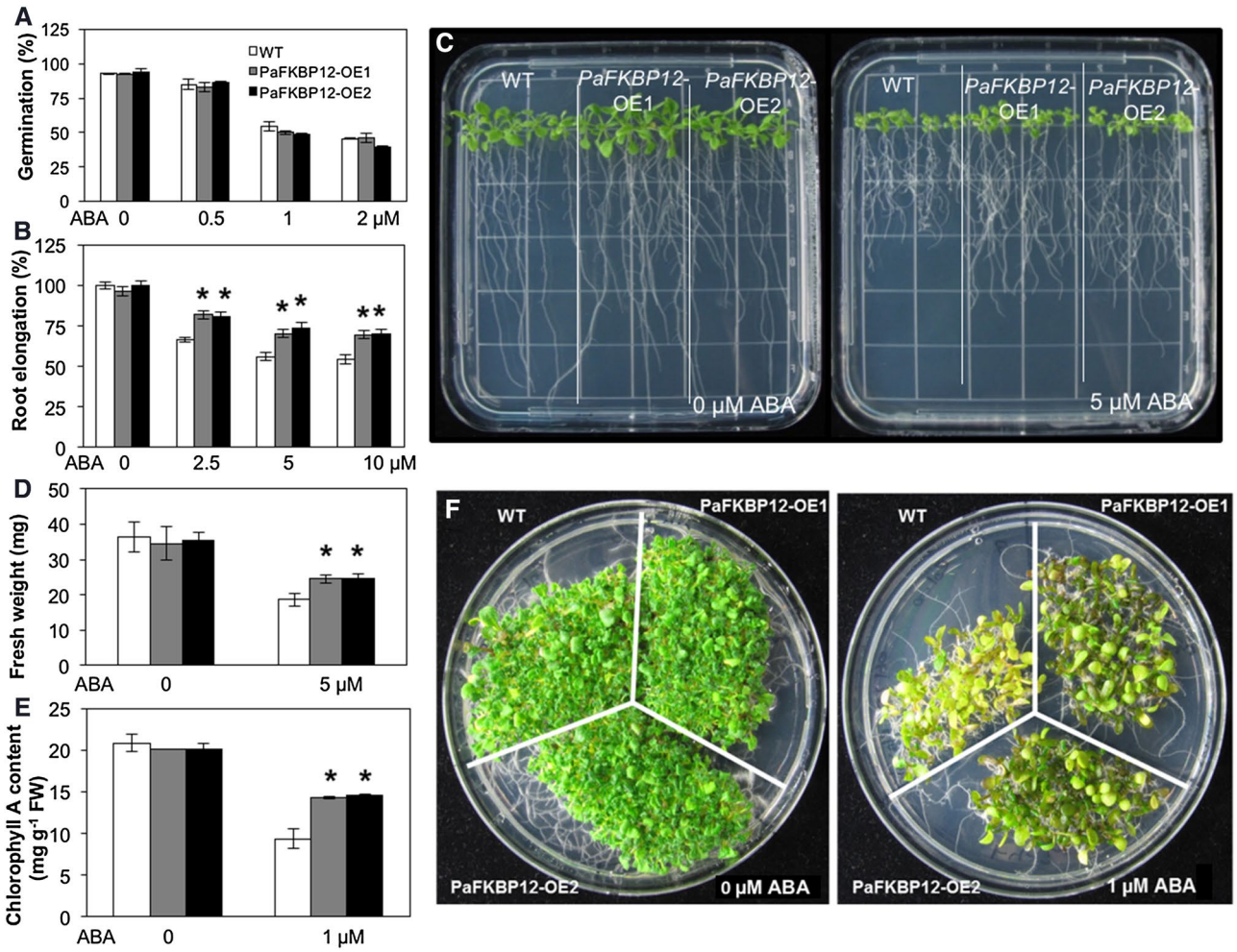

Fig. 5 Hyposensitivity to ABA in *PaFKBP12*-OE lines. **a** Quantification of seed germination WT and *PaFKBP12*-OE (3 days after ABA treatments). **b, c** Root elongation comparison between WT and *PaFKBP12*-OE at various ABA concentrations (8 days after transfer to ABA MS media from normal). **d** Fresh weight comparison between WT and *PaFKBP12*-OE at 5 µM ABA concentration (12 days). **e, f** Chlorophyll retention comparison of 18 day old seedlings between WT and *PaFKBP12*-OE on 0 or 1 µM ABA-containing MS media. Quantification of chlorophyll A contents (**e**) and seedling phenotypes (**f**). Error bars represent standard deviation of mean of three biological repeats. Asterisks indicate statistical significance in LSD test ($P < 0.05$)

Drought and salt stress tolerance in *PaFKBP12* overexpressing lines

ABA-hyposensitive phenotypes in *PaFKBP12*-OE at the seedling stage prompted us to examine the drought stress responses in *PaFKBP12*-OE because ABA is involved in drought responses (Kim 2014). To this end, 16-day-old WT and *PaFKBP12*-OE lines were left un-watered for 32 days, re-watered and allowed them to recover for 4 days. Under water deficit conditions for 32 days, only 18 out of 99 WT seedlings survived, whereas 33 out of 96 seedlings of *PaFKBP12*-OE1 and 64 out of 101 seedlings of *PaFKBP12*-OE2 remained alive (Fig. 6a, b). We also assessed the water retention capacity of *PaFKBP12* overexpressing lines by measuring the fresh weight in detached rosette leaves as described previously (Alavilli et al. 2016). During dehydration period, the fresh weights of WT rosette leaves were always less than those of *PaFKBP12*-OE leaves (Fig. 6c). These data showed that PaFKBP12 overexpression resulted in drought tolerance in plants.

ABA controls the stomatal movement (Kim et al. 2010). Thus, we tested if drought tolerance phonotype in *PaFKBP12*-OE is because of the stomatal opening status in *PaFKBP12*-OE different from that in WT. However, any remarkable differences in stomatal pore size between WT and *PaFKBP12*-OE lines were not observed either in the presence or absence of ABA (Suppl. Fig. 1).

In many cases, drought stress tolerance concurs with salt stress tolerance. Thus, we examined the salt stress tolerance in *PaFKBP12*-OE lines. In germination test, *PaFKBP12*-OE clearly germinated at higher rates than WT (Suppl. Fig. 2a). *PaFKBP12*-OE maintained 34–50% germination rate at 200 mM NaCl where WT germinated only approximately 13% (Suppl. Fig. 2a). In addition, survival rate and chlorophyll contents on high salt conditions were higher in *PaFKBP12*-OE than in WT (Suppl. Fig. 2b–e), which suggested enhanced stress tolerance in *PaFKBP12*-OE lines. Root elongation in *PaFKBP12*-OE appeared to be slightly higher than in WT although it was not significant (Suppl. Fig. 2f). Taken together, *PaFKBP12* overexpression increased drought and salt stress tolerance in *Arabidopsis*.

Expression of stress-responsive genes in *PaFKBP12*-OE lines

We next examined the stress-responsive gene expression in *PaFKBP12*-OE. It is well known that stress signals induce the expression of genes that are important for stress adaptation, which causes stress tolerance in plants (Park et al. 2016; Zhu 2002, 2016). We first measured the expression levels of two genes involved in heat stress tolerance (Hong

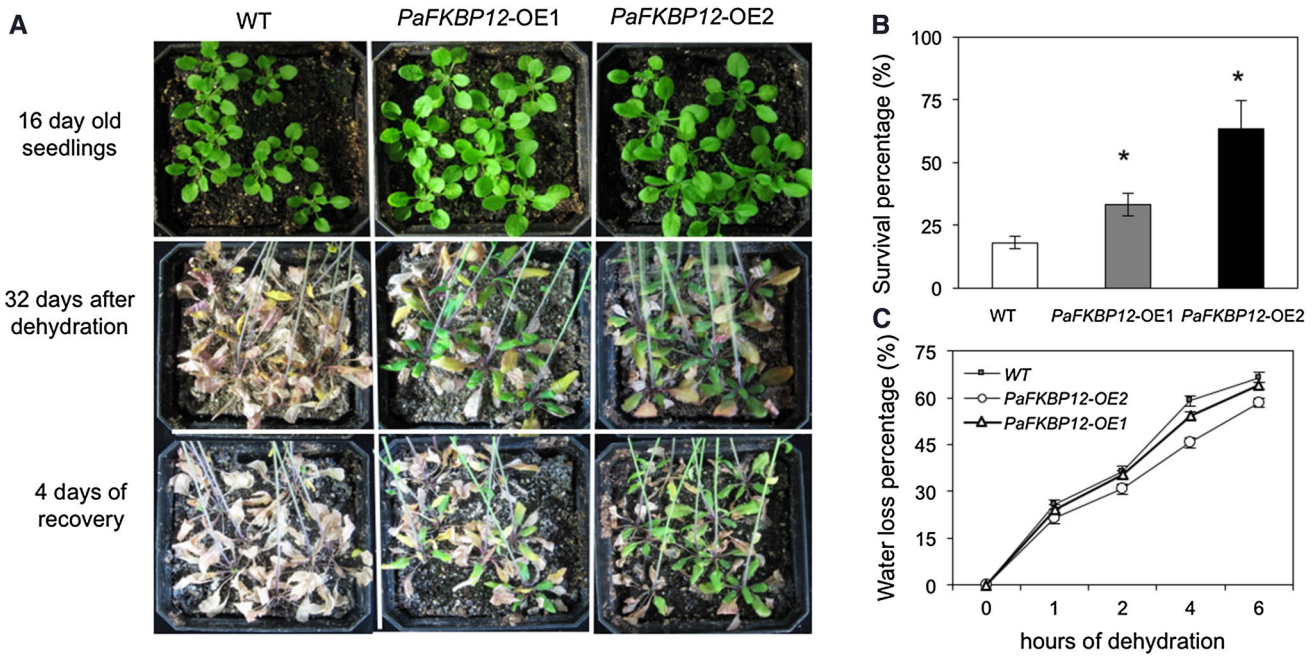

Fig. 6 Drought stress tolerance in *PaFKBP12*-OE lines. **a** Drought tolerance comparison between WT and *PaFKBP12*-OE (6–7 weeks old). Water was withheld for 32 days followed by 4 days of recovery. **b** Quantification of survival rates in WT and *PaFKBP12*-OE under drought stress (**a**). Survival rates were recorded after 4 days of stress recovery. **c** Water loss measurement in WT and *PaFKBP12*-OE. Fresh weights of the rosette leaves of WT and *PaFKBP12*-OE were measured at various time points after detachment from plant. Error bars represent standard deviation of mean of at least three biological repeats. Asterisks indicate statistical significance in LSD test ($P < 0.05$)

and Vierling 2000; Montero-Barrientos et al. 2010). After heat treatment (45 °C, 3 h), the levels of heat-tolerant *HSP70* and *HSP101* transcripts were significantly higher in *PaFKBP12*-OE than WT (Fig. 7a, b). *KIN1* and *RAB18* are the genes induced by multiple stresses including ABA and drought, and are linked to stress tolerance upon high induction by stress (Zhu 2002, 2016). The two genes were highly induced by ABA (100 µM, 3 h) or drought (3 h) in both WT and *PaFKBP12*-OE, but the expression levels were much higher in *PaFKBP12*-OE than in WT (Fig. 7c–f). Similarly, the expressions of other stress-responsive genes such as *RD20* (At2g33380), *DREB2A* (At5g05410), *COR15A* (At2g42540), *RD29B* (At5g52300), but not *NCED3* (At3g14440) were higher in *PaFKBP12*-OE than in WT under ABA treatment or drought stress (Suppl. Fig. 3). Taken together, these results suggested that enhanced stress tolerance in *PaFKBP12*-OE might, at least in part, be attributed to increased induction of stress-responsive genes.

Discussion

Fortifying crop plants with multiple stress tolerance is a key solution to improve the crop yield for the increasing global food demands. In this study, we found that the constitutive expression of *PaFKBP12* in *Arabidopsis* enhanced the plant tolerance to multiple abiotic stresses including heat, ABA, drought, and salt stress.

Phylogenetic analysis of PaFKBP12 with other species including animal and plant FKBP12 protein sequences found clear divergence between animal and plant species (Fig. 1). Also, within the plant FKBP12 clade there was a clear difference seen between the vascular and non-vascular plants (Fig. 1). This suggests that FKBP12 proteins across the species potentially contain distinct functions despite they all contain one single FKB conservative domain. Therefore, heterologous expression of FKBP12

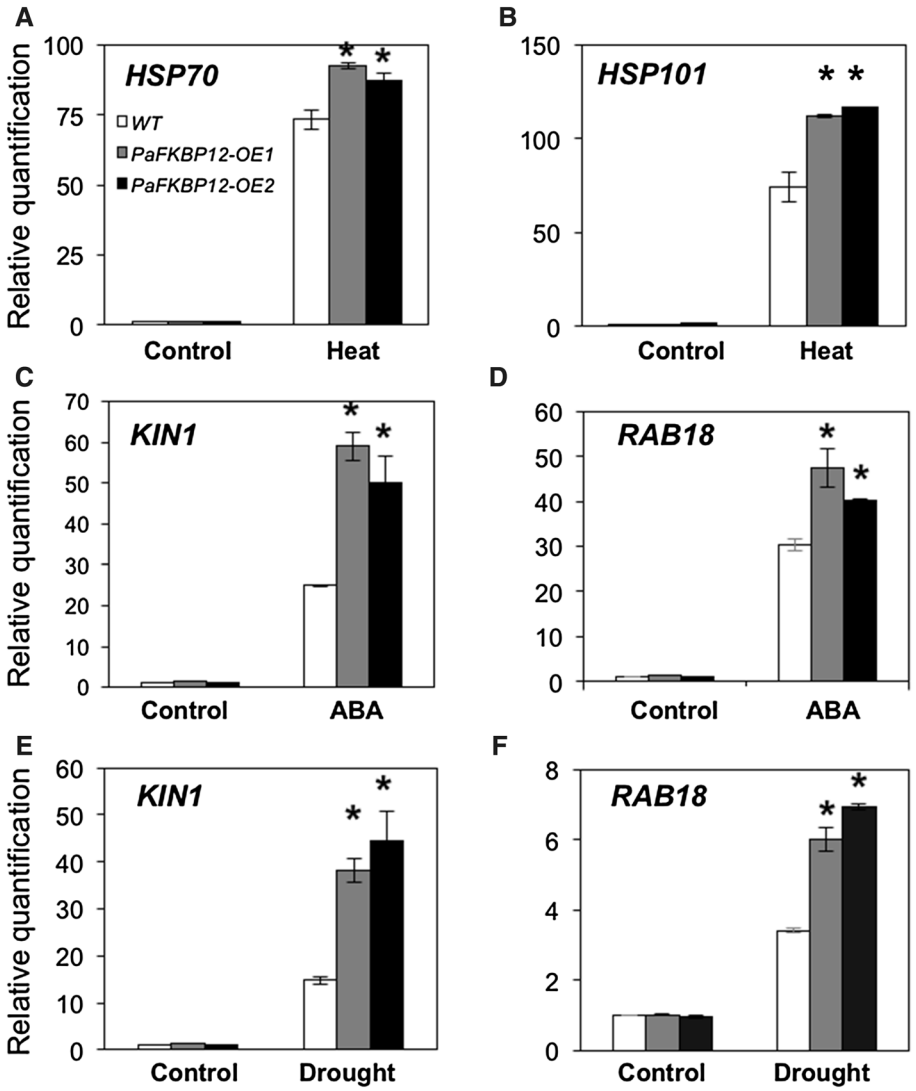

Fig. 7 Stress inducible gene expression in *PaFKBP12*-OE lines. Relative expressions of stress inducible genes in WT and *PaFKBP12*-OE in response to heat (45 °C for 3 h), ABA (100 µM for 3 h) and drought (air dry on a filter paper for 3 h) were measured by qRT-PCR. Arabidopsis *Clathrin* (At4g24550) gene was used as an internal control for normalization of different cDNA samples. Three biological replicates were averaged and error bars represent standard deviation

could bring about new traits that cannot be achieved by the endogenous gene modulation. Indeed, our previous study on *Multiprotein Bridging Factor 1c* (*MBF1c*) from *P. alpinum* showed neofunctionalilty of *MBF1c* (Alavilli et al. 2017). In the study, *PaMBF1c* overexpressing *Arabidopsis* displayed higher tolerance to salt and ionic stresses than *Arabidopsis* overexpressing the endogenous *MBF1c* (AtMBF1c), while the degrees of heat stress tolerance in both lines were similar to each other (Alavilli et al. 2017).

Another characteristic of *PaFKBP12* different from *AtFKBP12* is its stress inducibility. *AtFKBP12* is known to be ubiquitously expressed and not affected by stress (Geisler and Bailly 2007), while *PaFKBP12* was ABA and heat-induced (Fig. 2). Very recently, *FKBP12* from thermophilic microalga, *Scenedesmus* sp. was shown to be induced by heat and salt stress and brought about tolerance to each stress when overexpressed in *E. coli* (Subin et al. 2016). In our study, we found the *PaMBF1c* overexpression increased heat, ABA, drought, and salt stress tolerance in *Arabidopsis*. At this moment, stress phenotypes of *AtFKBP12* overexpressing plants have not been reported. Thus, detailed comparisons should be carried out to understand better the differences in functions of *PaFKBP12* and *AtFKBP12*. Nonetheless, it is tempting to claim that circumstantial evidence (see above) still argue that differences in amino acid residues and/or transcriptional regulation in *PaFKBP12* might have led to enhanced stress tolerance in *PaFKBP12*-OEs. In addition, *AtFKBP12* mutant did not show distinctive phenotypes in development (Gollan et al. 2012; Vespa et al. 2004), while we found that *PaFKBP12*-OE displayed bigger sized leaves than WT. Our cell size comparison suggested that the enlarged leaves could be because of rapid cell cycle that caused small-sized cells in leaves. It has been shown that the mice deficient in *FKBP12* resulted in cell cycle arrest in G1 phase (Aghdasi et al. 2001), consistently supporting our findings.

Although our *PaFKBP12*-OEs tolerance phenotypes after ABA treatment and NaCl treatment were similar at the seedling stage, germination rates of *PaFKBP12*-OE were higher than WT only under salt stress, not at ABA treatments (Fig. 5a and Suppl. Fig. 2a). These results indicate that *PaFKBP12*-mediated sensitivity to ABA and NaCl during germination and during post-germination growth could be different.

Expressions of stress-responsive genes were closely related to stress tolerance (Zhu 2002, 2016). In our gene expression analysis, we found that most genes we tested were highly up-regulated by heat, ABA and drought stress in comparison with WT (Fig. 7 and Suppl. Fig. 3). Thus, increased induction of these stress-responsive genes might, at least in part, contribute to the enhanced stress tolerance in *PaFKBP12*-OE. Our results also suggested that *PaFKBP12* is likely to act upstream of these stress inducible genes, but not to interfere with ABA biosynthesis upon ABA treatment because the induction level of ABA biosynthetic *NCED3* gene was not different between WT and *PaFKBP12*-OE (Suppl. Fig. 3b). We also noticed that the stress responsible gene expression levels in *PaFKBP12*-OE under normal conditions were not very much different from the ones in WT. Thus, it is likely that *PaFKBP12* mainly functions after stress signal is initiated. It should be noted that the enlarged leaves were observed under normal conditions, not under stress conditions only. Therefore, these results suggest that regulations of *PaFKBP12* by developmental and stress signals would be different.

Many gene overexpression studies demonstrated that the overexpression-induced ABA hypersensitivity is correlated to enhanced drought tolerance (Kim et al. 2014; Li et al. 2017; Park et al. 2011). Given the fact that ABA is induced by drought stress, this seems logical. However, there are also some opposite reports, including this study, that showed improved drought tolerance associated with overexpression-caused ABA hyposensitivity. For example, *Arabidopsis* overexpressing a group A PP2C from rice (*OsPP108*) showed high insensitivity to ABA along with high-tolerance to salt, mannitol and drought stress (Singh et al. 2015). Also, overexpression of a pepper lipoxygenase *CaLOX1* resulted in ABA insensitivity and enhanced tolerance to osmotic, drought and salt stress (Lim et al. 2015). Thus, similar to these observations, the *PaFKBP12* overexpression might achieve drought tolerance via ABA-independent ways. However, it should be noted that *PaFKBP12* overexpression does not likely cause the systemic ABA-insensitivity throughout the whole body. Indeed, *PaFKBP12*-OE was not ABA-hyposensitive in germination and stomatal closure (Fig. 5a and Suppl. Fig. 1). Additionally, *PaFKBP12*-OE was hypersensitive to ABA in induction of *KIN1, RAB18* and *RD20* genes (Fig. 7c, d and Suppl. Fig. 3a). Therefore, at least the induction of these stress-responsive genes might contribute to the drought tolerance in *PaFKBP12*-OE through ABA-dependent ways. These fluctuations of ABA sensitivity in *PaFKBP12*-OE throughout the plant life cycle and body suggest development and tissue-specific ABA signaling and modulations. G-proteins in ABA signaling is in agreement with this notion; G-proteins are known to regulate ABA-dependent stomatal responses positively and modulate ABA-dependent germination and growth negatively (Fan et al. 2008; Pandey et al. 2006; Wang et al. 2001).

In summary, we uncovered important functions in *PaFKBP12* in development and stress using *PaFKBP12* overexpressing *Arabidopsis* lines. In particular, multiple stress tolerance in *Arabidopsis* by *PaFKBP12* overexpression suggest that a genetic resource from extremophytes could be useful in improving stress tolerances in agriculturally important plants.

Author contribution statement HL, DJY and BhL conceived and designed research. HA and MP conducted experiments. HA, HL, MP, DJY and BhL analyzed data. HA, HL, DJY and BhL discussed the results. HA and BhL wrote the manuscript.

Acknowledgements This work was supported by the Global Research Laboratory Program (2017K1A1A2013146) through the National Research Foundation (NRF) funded by the Ministry of Science and ICT (MSIT), Republic of Korea, and by Polar Genomics 101 Project (PE17080), the Korea Polar Research Institute.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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