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Calmodulin-binding protein phosphatase PP7 is involved in thermotolerance in *Arabidopsis*

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ABSTRACT

PP7 is the first protein Ser/Thr phosphatase to be found to interact with calmodulin (CaM) in plants. The T-DNA insertion *AtPP7* knockout line and *AtPP7* overexpression lines were employed to study the specific function of AtPP7. The *AtPP7* knockout impaired the thermotolerance of *Arabidopsis* seedlings while the overexpression of *AtPP7* resulted in plants with increased thermotolerance. Results from real-time polymerase chain reaction (PCR) showed that the expression of *AtHSP70* and *AtHSP101* genes was up-regulated in *AtPP7* overexpression lines after heat shock (HS) at 37 °C for 1 h. Protein gel blot analysis showed that HSP70 protein levels increased in *AtPP7* overexpression lines after HS at 37 °C for 2 h. The expression of the *AtPP7* gene was also induced by HS at 37 °C in wild-type *Arabidopsis*. Using a yeast two-hybrid screen, we showed an interaction between AtPP7 and CaM. In addition, we found that AtPP7 interacts with an HS transcription factor (HSF), suggesting a possible role for AtPP7 in regulating the expression of heat shock protein (HSP) genes.

Key-words: AtPP7; HSF; HSP.

Abbreviations: CaM, calmodulin; HS, heat shock; HSF, heat shock transcription factor; HSP, heat shock protein.

INTRODUCTION

Phosphorylation and dephosphorylation of proteins catalysed by protein kinases and protein phosphatases often serve as 'on-and-off' switches in the regulation of many cellular activities (Luan 2003). Although protein kinases and phosphatases both play critical roles in cellular signal transduction, it is protein kinases that have been more widely studied experimentally (Droillard *et al*. 2004; Nakagami, Kiegerl & Hirt 2004). Some studies about the functions of protein phosphatases also have been carried out (Bellec *et al*. 2002; Kwak *et al*. 2002; Petters *et al*. 2002). These results have clearly demonstrated that protein phosphatases function not only by counterbalancing the protein kinases but also by taking a leading role in many signalling events (Luan 1998).

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Calcium signalling mechanisms are widely employed by all eukaryotic organisms to regulate gene expression and a variety of other cellular processes. Ca^{2+} mediates intracellular signalling mainly through activating Ca^{2+}/CaM-dependent protein kinases and phosphatases (Clapham 1995; Hunter 1995; Trewavas *et al*. 1996). A major effector of Ca^{2+}/CaM signalling in animals and fungi is the Ca^{2+}/CaM-dependent protein Ser/Thr phosphatase calcineurin (Guerini 1997; Kudla *et al*. 1999). However, there is no sequence data so far that would confirm the existence of homologs of a calcineurin catalytic subunit in plants (Kutuzov, Bennett & Andreeva 2001), although 10 calcineurin regulation subunit-like proteins have been identified from *Arabidopsis thaliana* and cloned (Luan *et al*. 2002).

Arabidopsis thaliana PP7 cDNA encoding a novel protein Ser/Thr phosphatase has been described. While PP7 is a member of the PPP family, it is not closely related to any protein phosphatases in animals or fungi (Andreeva *et al*. 1998; Kutuzov, Evans & Andreeva 1998). The complete *PP7* sequence is available to date only for *A. thaliana*, but partial sequences from some other plant species, including maize, rice, etc., indicate that PP7 may be present throughout the plant kingdom (Andreeva *et al*. 1997, 1998; Andreeva & Kutuzov 1999). *Arabidopsis thaliana* PP7 (AtPP7) possesses a CaM-binding site at the first insert in its catalytic domain. Subsequent biochemical studies have demonstrated that recombinant AtPP7 is able to specifically interact with CaM in a Ca^{2+}-dependent manner (Kutuzov *et al*. 2001). *AtPP7* transcript can be detected in all *A. thaliana* tissues with reverse transcriptase mediated polymerase chain reaction (RT-PCR) and Northern blot analysis. However, the highest levels of *AtPP7* expression are in a subset of stomata (Andreeva & Kutuzov 1999). Moreover, AtPP7 has been shown to be a nuclear protein (Andreeva & Kutuzov 2001; Moller *et al*. 2003). The interaction with CaM, as well as the AtPP7 resistance to okadaic acid, suggests that AtPP7 might be responsible for the 'calcineurin-like' phosphatase activity in plants (Kutuzov *et al*. 2001). It was reported that AtPP7 acts as a positive regulator of cryptochrome signalling in *A. thaliana* (Moller *et al*. 2003).

In recent years, the role of the Ca^{2+}-CaM signal system in regulating the expression of HSP genes has been investigated in our laboratory (Li *et al*. 2002). Our observations indicated that intracellular Ca^{2+} levels increased during HS in wheat seedlings (Liu *et al*. 2003) and *A. thaliana*

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suspension cells (Liu *et al*. 2006). Furthermore, the levels of CaM mRNA and protein increased during HS in the presence of Ca^{2+}, and Ca^{2+} and CaM together regulate the expression of HSP genes and synthesis of HSPs (Fan *et al*. 2000; Liu *et al*. 2003; Liu, Sun & Zhou 2005). The modulation of HSF DNA-binding activity by Ca^{2+}-CaM *in vitro* has been previously demonstrated (Li *et al*. 2004). Based on our findings, we have proposed a pathway for the participation of Ca^{2+}-CaM in HS signal transduction (Liu *et al*. 2003), although the specific mechanism by which CaM regulates the activation of HSF is not known.

AtPP7 is the first plant protein Ser/Thr phosphatase to be found to interact with CaM (Kutuzov *et al*. 2001), and therefore a good candidate for regulating the activity of HSF in the Ca^{2+}/CaM pathway. We focus on the role of AtPP7 in the HS response in this paper. Based on the results presented herein, we suggest a possible function of AtPP7 in activating the expression of HSP genes.

MATERIALS AND METHODS

Plant materials and growth conditions

Seeds of *A. thaliana* (ecotype Columbia) were surface-sterilized, sown onto MS medium (Murashige & Skoog 1962) containing 1.0% (w/v) sucrose and 0.8% agar, and kept at 4 °C for 3 d. Plants were then cultured to the flowering stage in growth chambers at 22 °C and ~ 100 μmol photons m^{-2} s^{-1} with a 16 h light cycle.

Three T-DNA insertion lines (SALK 047299, SALK 089764 and SALK 060026) for AtPP7 (At5g63870) were obtained from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, USA). Only one of the T-DNA insertion lines (SALK 089764) showed expression of the *AtPP7* gene was completely suppressed. The homozygous mutant line was named *pp7* and used for further analysis.

Real-time PCR

Primer pairs for real-time PCR were designed using Primer Express (Applied Biosystems, Foster City, CA, USA) (Table 1). Gene-specific primers were chosen so that the resulting PCR products were in the range of 80–130 bp. Two micrograms of total RNA was treated with Dnase I and then was used for first-strand cDNA synthesis using Oligo d(T) primers and Taqman Reverse Transcription Reagents (Applied Biosystems) at 25 °C for 10 min, then 48 °C for 30 min and 95 °C for 5 min. For real-time PCR, the reaction mixture consisted of cDNA first-strand template, primer mix (5 μM) and SYBR Green Master Mix (Applied Biosystems) in a total volume of 25 μL. The following programme was applied: initial polymerase activation at 95 °C, 10 min; 40 cycles of 95 °C, 15 s/60 °C, 60 s. The reaction was performed using an ABI prism 7000 Sequence Detection System (Applied Biosystems). Actin was used as the internal control. The data were analysed using Microsoft Excel (Microsoft, Redmond, WA, USA). Each datum point is the mean value from biological triplicate determinations.

Analysis of *AtPP7* expression by RT-PCR

AtPP7 Transcript abundance in wild-type and T-DNA insertion plants was determined by RT-PCR. Denatured total RNA (1 μg) plus the forward (5′-CGGAATTCT TAATGGAAACTGTTCCACC-3′) and reverse (5′-CC GCTCGAGGTCAGCTATTTGGTTGTTCG-3′) primers were used with the One Step RNA RT-PCR kit (Takara, Otsu, Shiga, Japan). RT-PCR proceeded at 50 °C for 30 min, then 94 °C for 2 min, 30 cycles of 94 °C, 30 s/55 °C, 30 s/ 72 °C, 2 min, followed by a final extension at 72 °C for 10 min. The *actin* transcript was used as the internal control, with the forward (5′-AGGCACCTCTTAACCCTAAAGC-3′) and reverse (5′-GGACAACGGAATCTCTCAGC-3′) primers.

Vector construction and plant transformation

The AtPP7 coding region was amplified by PCR from *AtPP7* cDNA with the forward (5′- GCTCTAGAAAC AATGGAAACTGTTCCACC-3′) and the reverse (5′-GCGTCGACTCAGCTATTTGGTTGTTC-3′) primers. The PCR product was cloned into the binary vector pCAMBIA1300 which contains the 35S promoter of *Cauliflower mosaic virus* with *XbaI* and *SalI*. The construct was transformed into the *Agrobacterium tumefaciens* strain GV3101. The pCAMBIA1300-35S : AtPP7 was introduced into the wild-type plants by the floral dip method (Clough & Bent 1998). Transformants were selected on MS medium containing 25 μg/mL of hygromycin. Resistant seedlings were

Table 1. Primers used for real-time polymerase chain reaction

Target genes	Accession no.	Primer sequences
AtPP7	At5g63870	5′-CTGGACGTGTTTATACAGCACATG-3′ 5′-CTTGTTGTTCGCTTAGGCAAGA-3′
AtHSP70	At3g12580	5′-AACCGCACCACTCCTTCCTA-3′ 5′-GGGTTCATGGCGACTTGATT-3′
AtHSP101	At1g74310	5′-GCAGCTTCGATGCATTGGT-3′ 5′-TGGCTCCGCAACATAGACTTG-3′
ACTIN	AY074873	5′-TGTGCTCAGTGGTGGAACCA-3′ 5′-GGAGCCAAAGCAGTGATCTCTT-3′

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transplanted to soil and grown in a greenhouse to produce seeds. Homozygous transformants were used in all analyses.

Protein gel blot analysis

Fourteen-day-old seedlings were treated at 37 °C for 2 h. Three hundred milligrams of treated plant material was ground in liquid N_2, mixed with 600 μL of extraction buffer (10 mM Hepes, pH 7.9, 0.4 M NaCl, 0.5 mM dithioreitol (DTT), 0.1 mM glycol-bis-(α-aminoethyl)ether N,N,N,N-tetraacetic acid (EGTA), 5% glycerin, 0.5 mM phenylmethylsulfonyl fluoride), then clarified by centrifugation. The supernatant was used as a protein sample. Per sample 30 μg of total protein was separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970; Schagger, Aquila & Jagow 1988), followed by electrotransfer to nitrocellulose membranes. The blots were incubated for 12 h at room temperature in phosphate-buffered saline (PBS) containing 5% non-fat dry milk, and then incubated for 2 h at room temperature in PBST (PBS including tween-20) containing the appropriate antibody. Secondary anti-rabbit antibody (Sigma-Aldrich, St Louis, MO, USA) conjugated to alkaline phosphatase was used for immuno-detection. Tubulin was used as a loading control.

Assay of thermotolerance

Seeds were plated on MS plates (1% sucrose) and cultured as described earlier for 10 d. Plates were exposed to 45 °C for 30 or 45 min, and then allowed to recover at 22 °C. Seedling viability was monitored for up to 10 d, but typically results were documented after 7 d of recovery at 22 °C.

Yeast two-hybrid assays

The AtPP7 coding region was amplified using forward (5′-CGGAATTCTTAATGGAAACTGTTCCACC-3′) and reverse (5′-AACTGCAGGTCAGCTATTTGGTTGTT CG-3′) primers, then ligated with *EcoRI* × *PstI*–digested pGBKT7 (Clontech, Mountain View, CA, USA). The AtHSF1 (At4g17750) coding region was amplified using forward (5′-CATGCCATGGGTATGTTTGTAAATTTC-3′) and reverse (5′-CATGCCATGGCTAGTGTTCTG TTTCTGA-3′) primers, then ligated with *NcoI*-digested pGADT7 (Clontech). The AtCaM3 (At3g56800) coding region was amplified using forward (5′-CATGCCAT GGCAATGGCGGATCAGCTCAC-3′) and reverse (5′-GGAATTCCATCACTTAGCCATCATGACC-3′) primers, then ligated with *NcoI* × *EcoRI*-digested pGADT7 (or pGBKT7 for vector swapping). Expression vectors pGADT7-AtHSF1 (or pGADT7-AtCaM3) and pGBKT7-AtPP7 were introduced into the yeast strain AH109 (Clontech) by the lithium acetate method as described in the *Clontech Yeast Protocols Handbook*. In order to make the construct vector swapping, the AtPP7 coding region was amplified using forward (5′-CGGAATTCTTAATGG AAACTGTTCCACC-3′) and reverse (5′-CCGCTCG AGGTCAGCTATTTGGTTGTTCG-3′) primers, and then ligated with *EcoRI* × *XhoI*-digested pGADT7. AtHSF1 coding region was amplified using forward (5′-CATG CCATGGGTATGTTTGTAAATTTC-3′) and reverse (5′-AACTGCAGCTAGTGTTCTGTTTCTGA-3′) primers, then ligated with *NcoI* × *PstI*-digested pGBKT7. Expression vectors pGBKT7-AtHSF1 (or pGBKT7-AtCaM3) and pGADT7-AtPP7 were introduced into the yeast strain AH109. Cells were plated onto selective medium lacking Trp, Leu, then putative transformants were transferred to the medium lacking Trp, Leu, His and adenine. The activity of β-galactosidase using the substrate 5-bromo-4-chloro-β-D-galactosidase (x-Gal) was monitored with a colony-lift filter assay. The autoactivation of the plasmids was analysed by β-galactosidase assay.

RESULTS

Expression of the *AtPP7* gene under HS and in different genotypic plants

To better understand the function of AtPP7 in the HS response, the gene expression pattern of *AtPP7* was determined in wild-type *Arabidopsis* during HS. Total RNA was isolated from 10-day-old wild-type *Arabidopsis* seedlings heat shocked at 37 °C for 10, 20 or 30 min, respectively. real-time PCR was performed using a pair of primers specific to the *AtPP7* gene described in Table 1. The result showed that the expression of *AtPP7* increased after HS at 37 °C and remained elevated for at least 30 min (Fig. 1).

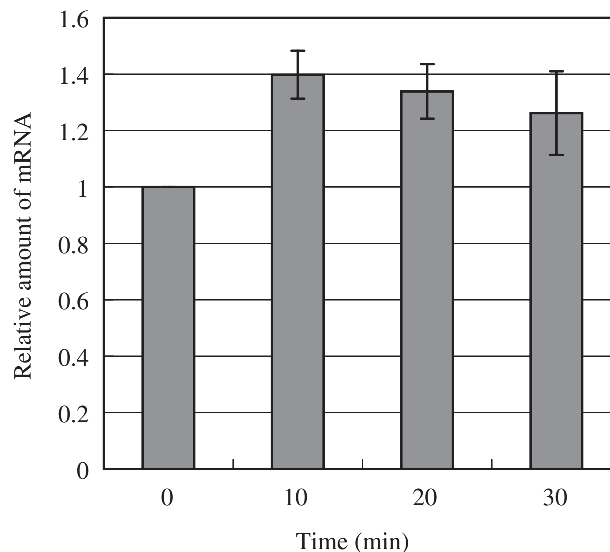

Figure 1. The expression of *AtPP7* gene induced by HS at 37 °C. Ten-day-old wild-type seedlings were heat shocked at 37 °C for 0, 10, 20 or 30 min, then total RNA was isolated respectively, converted into cDNA and subjected to real-time polymerase chain reaction using *AtPP7* gene-specific primers (Table 1). *Actin* was used as a control, and samples from non-HS seedlings were used as calibrators. Each datum is presented as mean ± SD from three independent experiments, and SD bars are shown.

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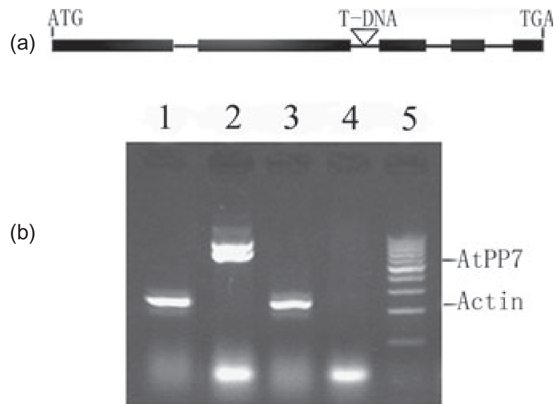

Figure 2. Reverse transcriptase mediated polymerase chain reaction (RT-PCR) analysis of *AtPP7* transcript in T-DNA insertion mutant of *AtPP7*. (a) Intron-exon organization of the *AtPP7* gene (coding region) and the location of T-DNA insert. Solid boxes and lines indicate exons and introns, respectively. The position of the T-DNA insert is indicated by a triangle. (b) RT-PCR analysis of *AtPP7* transcript in the T-DNA insertion mutant of *AtPP7*. The RT-PCR was performed using *actin* and *AtPP7* whole-sequence primers, respectively. The *actin* expression level was analysed as a control. Lane 1, the expression of the *actin* in wild-type seedlings; lane 2, the expression of the *AtPP7* in wild-type seedlings; lane 3, the expression of the *actin* in *pp7* mutant; lane 4, the expression of the *AtPP7* was suppressed in *pp7* mutant; lane 5, 200 bp DNA marker.

To genetically dissect the function of AtPP7, we screened the T-DNA insertion alleles of the *AtPP7* gene using lines deposited in the SALK Institute Genomic Analysis Laboratory (http://signal.salk.edu). Among the three SALK insertion lines obtained, only one (designated *pp7*) was usable. As shown in Fig. 2a, the T-DNA in *pp7* is inserted into the second intron. Analysis by RT-PCR revealed that in the mutant, the expression of *AtPP7* gene was completely suppressed compared with the wild type (Fig. 2b).

Transgenic *AtPP7* overexpression plants were prepared using the binary vector pCAMBIA1300-35S. The full-length *AtPP7* cDNA was used to construct binary vectors. A number of hygromycin-resistant plants were generated and selfed to obtain T2 plants. Twenty-five independent T3 homozygous transgenic lines having a single copy of *35S : AtPP7* were generated.

An obvious overexpression of the *AtPP7* gene in transgenic lines was observed using real-time PCR analysis. The expression of the *AtPP7* gene was not equal in different transgenic lines. Compared with the wild type, *AtPP7* overexpression lines 14-2, 17-3 and 109-8 had 4.3-, 11.4- and 6.9-fold increase, respectively, in *AtPP7* transcript levels (Fig. 3).

Effect of AtPP7 on the expression of HSP genes during HS

HSPs are induced by HS, and play an important role in the acquisition of plant thermotolerance (Queitsch *et al.* 2000; Burke 2001). To understand whether expression of the *AtPP7* gene affects the expression of HSP genes, by real-time PCR we analysed the expression of two HSP genes, *AtHSP70* and *AtHSP101*, in the wild-type and three independent *AtPP7* overexpression lines (14-2, 17-3, 109-8) after HS at 37 °C for 1 h. The expression of the *AtHSP70* and *AtHSP101* genes is induced by HS (Queitsch *et al.* 2000; Sung, Vierling & Guy 2001). After HS at 37 °C for 1 h, the expression of the *AtHSP70* gene in the three overexpression lines (14-2, 17-3, 109-8) was 1.1-, 1.7- and 1.5-fold higher, respectively, than that in the wild-type plants (Fig. 4a), while the expression of the *AtHSP101* gene showed 1.3-, 2.8- and 1.7-fold increase (Fig. 4b). The expression of the *AtHSP70* and *AtHSP101* correlated positively with the expression levels of the *AtPP7* gene in the different *AtPP7* overexpression lines.

The effect of AtPP7 on the levels of HSP70 proteins was also tested in wild-type and transgenic lines after HS at 37 °C. Fourteen-day-old wild-type seedlings and three independent transgenic *AtPP7* overexpression lines (14-2, 17-3, 109-8) were heat shocked at 37 °C for 2 h. Protein samples were separated by SDS-PAGE and analysed by Western blot. AtHSP70 was detected using anti-maize HSP70 antiserum. The result showed that the abundance of HSP70 proteins in the *AtPP7* overexpression lines was higher than that in the wild-type plants under HS conditions (Fig. 4c). This experiment was repeated more than three times with reproducible results.

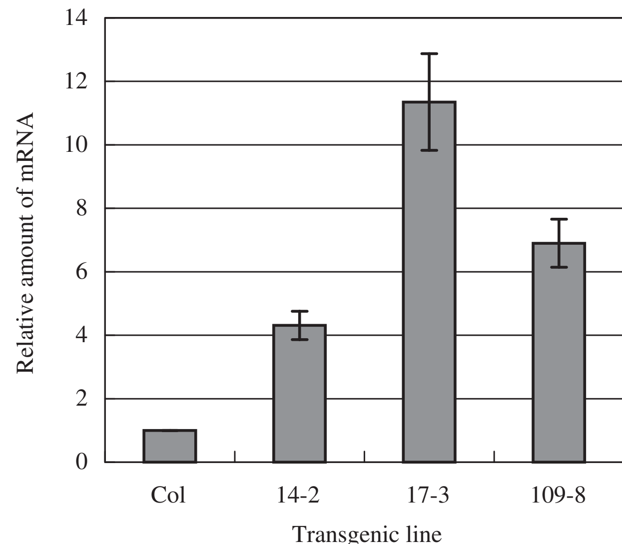

Figure 3. The expression of *AtPP7* gene in different transgenic lines. Total RNA was isolated from 10-day-old wild-type seedlings and three *35S* : *AtPP7* transgenic lines (14-2, 17-3, 109-8) respectively, converted into cDNA and subjected to real-time polymerase chain reaction using *AtPP7* gene-specific primers (Table 1). *Actin* was used as a control, and samples from wild-type seedlings were used as calibrators. Each datum point was the mean ± SD of three repeats, and SD bars are shown. Col, wild type; 14-2, 17-3 and 109-8, transgenic lines.

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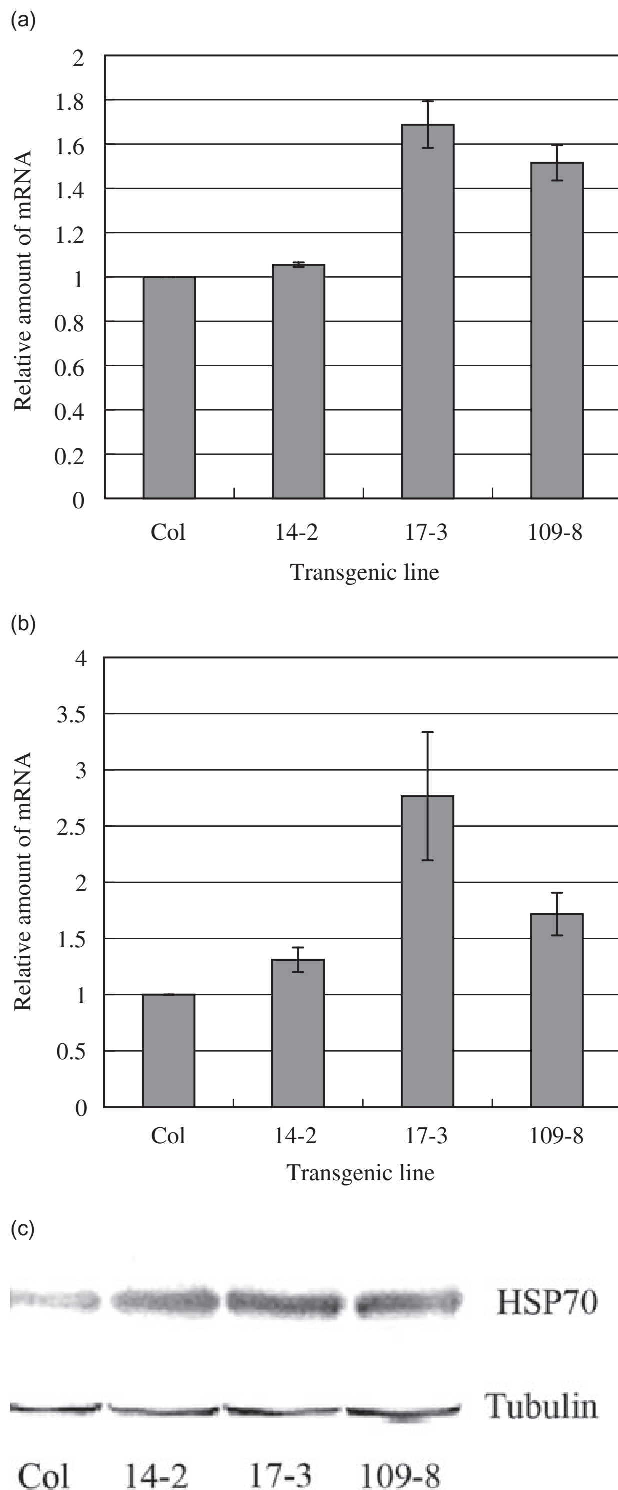

Figure 4. Expression of HSP genes in wild-type and *AtPP7* overexpression lines after HS at 37 °C. The expression of *AtHSP70* (a) or *AtHSP101* (b) gene in wild type and three *AtPP7* overexpression lines. Ten-day-old wild-type (Col) and three independent *AtPP7* overexpression line (14-2, 17-3, 109-8) seedlings were heat shocked at 37 °C for 1 h, and then total RNA was isolated respectively, converted into cDNA, and subjected to real-time polymerase chain reaction (PCR) using *AtHSP70* or *AtHSP101* gene-specific primers (Table 1). The real-time PCR conditions were the same as described in Fig. 3. (c) The abundance of HSP70 proteins in wild-type and *AtPP7* overexpression lines. Total proteins were separated by sodium dodecyl sulfate – polyacrylamide gel electrophoresis from 14-day-old wild-type (Col) and three *AtPP7* overexpression line (14-2, 17-3, 109-8) seedlings heat shocked at 37 °C for 2 h, then analysed by Western blot. The HSP70 proteins were detected using anti-maize HSP70 antiserum. Tubulin was included as the quantification control.

In summary, the expression levels of HSP mRNA and protein are linked to the *AtPP7* transcript levels in the transgenic *AtPP7* overexpression lines.

Involvement of AtPP7 in the thermotolerance of *Arabidopsis* seedlings

No significant phenotypic difference was observed between the *pp7* mutant and wild-type plants under normal growth conditions. The *pp7* mutant seedlings were then tested for differences in their responses to heat stress. The seeds of the *pp7* mutant and wild type were plated on the same MS medium plate. The 10-day-old seedlings were exposed to 45 °C for 30 min, and then returned to 22 °C. We found that the *pp7* mutant seedlings were impaired in thermotolerance. After HS at 45 °C, the *pp7* mutant seedlings could not survive or exhibited a significant delay in growth, but the wild-type seedlings could survive and grow normally (Fig. 5a).

No obvious phenotypic change was observed between *AtPP7* overexpression lines and the wild-type seedlings under normal growth conditions, further confirming that AtPP7 may not function in growth and development. The *AtPP7* overexpression lines were then tested for differences with the wild-type seedlings in their responses to HS. The *AtPP7* overexpression lines and wild-type seedlings were planted on the same MS plate (1% sucrose). The 10-day-old seedlings were exposed to 45 °C for 45 min, and then returned to 22 °C. As a result, 56% of the 25 independent *AtPP7* overexpression lines exhibited higher thermotolerance than the wild-type seedlings did. After exposure to 45 °C for 45 min, most of the wild-type seedlings died, but the *AtPP7* overexpression seedlings could survive under this lethal stress. The *AtPP7* overexpression lines 17-3 and 109-8 enhanced clearly, and line 14-2 enhanced slightly in thermotolerance compared with wild-type seedlings (Fig. 5b). Although the *AtPP7* overexpression seedlings exhibited a delay in growth to some extent after HS at 45 °C, most of them turned green and resumed growth after 5 d of recovery at 22 °C.

In summary, all the results indicated that CaM-binding protein phosphatase AtPP7 mediates the HS response and thermotolerance in *A. thaliana* by up-regulating the expression of HSP genes.

Interaction of AtPP7 with AtHSF1 or AtCaM3

HSFs are the terminal components of a signal transduction pathway mediating the response to HS. Because the activity

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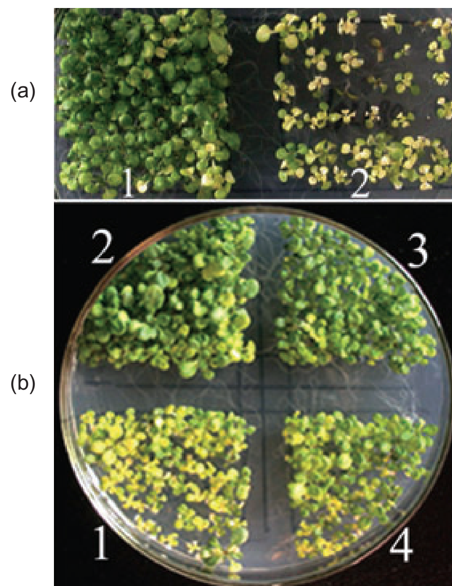

Figure 5. Effect of AtPP7 on the thermotolerance of *Arabidopsis* seedlings. (a) Knockout of *AtPP7* impairs the thermotolerance of *Arabidopsis* seedlings. Ten-day-old seedlings grown at 22 °C were exposed to 45 °C for 30 min, then returned to 22 °C. The plants were photographed on day 7 after being returned to 22 °C. 1 = wild type; 2 = *pp7* mutant. (b) Overexpression of *AtPP7* in three transgenic *AtPP7* lines improves thermotolerance. Ten-day-old seedlings grown at 22 °C were exposed to 45 °C for 45 min, then returned to 22 °C. The seedlings were photographed on day 7 after being returned to 22 °C. Most of the wild-type seedlings died after HS. The *AtPP7* overexpression lines 17-3 and 109-8 enhanced clearly, and 14-2 enhanced slightly in thermotolerance compared with the wild type. 1 = wild type; 2–4 = transgenic lines 17-3, 109-8, 14-2.

of HSF is regulated by phosphorylation, it is possible that AtPP7 affects the regulation. To confirm this possibility, we evaluated the ability of AtPP7 to interact with AtHSF1 by yeast two-hybrid assay. The capacity of the yeast strain AH109 to grow on synthetic medium lacking Trp, Leu, His and adenine, and β-galactosidase activity were used as the interaction reporters. We found that His auxotrophy was restored when *AtHSF1* was cotransformed with *AtPP7* (Fig. 6a). The interaction between AtHSF1 and AtPP7 was also tested by X-Gal filter assay and showed positive reaction (Fig. 6b). The interaction between AtHSF1 and AtPP7 suggested the possible role of AtPP7 in regulating the activity of HSF.

AtPP7 is a protein Ser/Thr phosphatase, which interacts with CaM (Kutuzov *et al.* 2001). Here, the *in vivo* interaction between AtPP7 and AtCaM3 was detected by yeast two-hybrid assay. In this assay, the yeast strain AH109 could not grow on synthetic medium lacking Trp, Leu, His and adenine, when *AtPP7* was cotransformed with *AtCaM3* (Fig. 6a). However, the interaction between AtCaM3 and AtPP7 was positive by X-Gal filter assay (Fig. 6b), indicating a weak or instantaneous interaction between AtPP7 and AtCaM3. The yeast strain AH109 did not show blue reaction by X-Gal filter assay when *AtPP7*, *AtHSF1* and *AtCaM3* were respectively cotransformed with *pGADT7* empty vector, showing that AtCaM3, AtPP7 and AtHSF1 do not have autoactivation (Fig. 6c).

With vector swapping, the same results for the interaction between AtPP7 and AtHSF1 or AtCaM3 were obtained (data not shown).

DISCUSSION

PP7 is a novel protein Ser/Thr phosphatase, not closely related to any other protein phosphatases in animals or fungi (Kutuzov *et al.* 1998). Moller *et al.* (2003) indicated that PP7 is a positive regulator of blue light signalling in *Arabidopsis*. Herein we report the role of AtPP7 in heat adaptation. No significant phenotypic difference was observed among the *pp7* mutant, *AtPP7* overexpression lines and the wild-type plants under normal growth conditions. After HS, the thermotolerance of *pp7* mutant seedlings was weaker than that of the wild type, and the thermotolerance of *AtPP7* overexpression lines was much stronger than that of the wild type (Fig. 5). Do the changes in levels of *AtPP7* mRNA lead to the observed changes in thermotolerance? The results presented herein show that the alteration in thermotolerance is related to the change in expressional levels of the *AtPP7* gene in the T-DNA insertion mutant and the multiple transgenic overexpression lines (Figs 2b, 3 & 5).

How does the expression of the *AtPP7* gene affect the thermotolerance of seedlings? Cells synthesize HSPs in

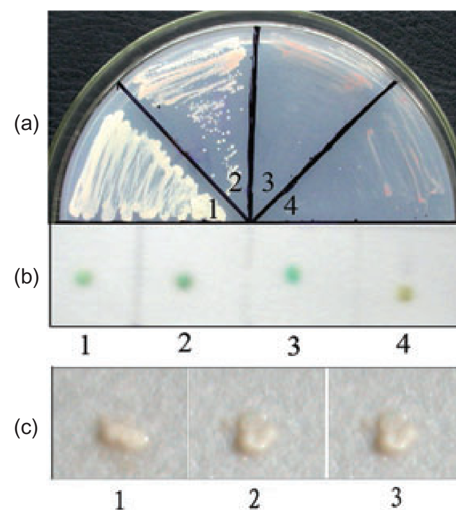

Figure 6. *In vivo* interaction between AtPP7 and AtHSF1 or AtCaM3. (a) Test of positive yeast clones on SD/Trp-Leu-His-Ade medium. 1 = positive control; 2 = pGADT7-*AtHSF1*/ pGBKT7-*AtPP7*: positive reaction; 3 = pGADT7-*AtCaM3*/ pGBKT7-*AtPP7*: negative reaction; 4 = negative control. (b) Results of β-galactosidase activity assay. 1 = positive control; 2 = pGADT7-*AtHSF1*/ pGBKT7-*AtPP7*: positive reaction; 3 = pGADT7-*AtCaM3*/ pGBKT7-*AtPP7*: positive reaction; 4 = negative control. (c) Test of autoactivation. *AtPP7*, *AtHSF1* and *AtCaM3* were respectively cotransformed with *pGADT7* empty vector. All show the negative reaction. 1 = AtPP7; 2 = AtHSF1; 3 = AtCaM3.

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response to heat, as well as other environmental stresses. HSP70 and HSP101 are important members of the HSP family. The expression of the *AtHSP70* and *AtHSP101* is induced by HS. Based on the expression pattern analysis of the *Arabidopsis HSP70* gene family, the response of the *AtHSP70* (At3g12580) gene to heat stress is the strongest of all cytosolic *AtHSP70*s (Sung *et al*. 2001). *AtHSP101* (At1g74310) plays a crucial role in a plant's ability to survive after heat stress (Queitsch *et al*. 2000). Thus, we chose *AtHSP70* and *AtHSP101* to evaluate the role of the AtPP7 in increasing the expression of HSPs. The results showed that the overexpression of *AtPP7* gene resulted in an increase in *AtHSP70* or *AtHSP101* expression and the abundance of HSP70 proteins in *Arabidopsis* after HS. The expression of the *AtHSP70* or *AtHSP101* gene is linked to the expression of the *AtPP7* gene in a dose-dependent fashion (Fig. 4).

In summary, there is a dose-dependent relationship between the expression of the *AtPP7* gene, the expression of *AtHSP70* or *AtHSP101* genes and thermotolerance of seedlings. Southern blot test showed that only a single copy of the *AtPP7* gene was inserted into the genome in the detected three transgenic lines (Supplementary Fig. S1). The results suggest that AtPP7 enhances the thermotolerance of *Arabidopsis* seedlings, and is also involved in the regulation of the expression of HSP genes. The AtPP7 affects thermotolerance through regulating the expression of multiple HSP genes contributing to thermotolerance in different extent. Together, these results support the proposal that AtPP7 is involved in the HS signal transduction in *Arabidopsis*.

The expression of HSP genes is mediated by HSFs. The reversible phosphorylation of HSFs is a key step in regulating their activity. The phosphorylation of HSFs involves distinct protein kinases/phosphatases and multiple phosphorylation sites (Kline & Morimoto 1997; Holmberg *et al*. 2001, 2002; Guettouche *et al*. 2005). The activity of HSFs can be activated by phosphorylation in some sites, or activated by dephosphorylation in other sites (Hoj & Jakobsen 1994). Holmberg *et al*. (2001) reported that human HSF1 could be phosphorylated by CaMK II on Ser230 and that CaMK II overexpression enhanced both the level of *in vivo* Ser230 phosphorylation and transactivation of HSF1, while Soncin *et al*. (2000) showed that an inhibitor of CaMK II enhanced the activity of human HSF1. A total of 21 open reading frames in the *Arabidopsis* genome were identified as encoding presumptive HSFs (Nover *et al*. 2001). Lower expression of AtHSF1 impairs both basal and acquired thermotolerance (Wunderlich, Werr & Schöffl 2003). The regulation of AtHsfA2 on the expression of heat stress genes (Schramm *et al*. 2006) and tolerance to heat and oxidative stress (Li *et al*. 2005) in *Arabidopsis* was also documented. Among at least 17 members in the tomato HSF family, a homolog of AtHSF1, LpHsfA1 has a unique role as a master regulator of thermotolerance (Mishra *et al*. 2002). HSF-mutant analysis showed that no obvious effects on HS response were observed in *hsf1* or *hsf3* individual mutant lines, only the *hsf1/hsf3* double mutant was significantly impaired in HS gene expression, suggesting that the genetic redundancy of class A-HSFs in *Arabidopsis* can compensate for the lack of a single HSF (Lohmann *et al*. 2004). It is undoubted that AtHSF1 is one of the master regulators for the expression of HSPs and thermotolerance. Thus, we chose AtHSF1 as a target gene of AtPP7 in this experiment. To know the possible role of AtPP7 in regulating HSF activity, we assessed the ability of AtPP7 to interact with AtHSF1 by yeast two-hybrid assays. The results showed that AtPP7 could interact with AtHSF1 *in vivo* (Fig. 6a,b), providing the possibility for AtPP7 to regulate the activity of HSF. Further experiments are ongoing.

AtPP7 possesses a CaM-binding site, and specifically interacts with CaM in a strict Ca^{2+}-dependent manner (Kutuzov *et al*. 2001). Yeast two-hybrid assays were employed to further study the *in vivo* interaction of AtPP7 with CaM. Among nine CaM genes from *Arabidopsis* (AtCaM1 to AtCaM9), the expression of *AtCaM3* was shown to increase intensely preceding the expression of HSP genes under HS conditions (Luan *et al*. 2002; Liu *et al*. 2005). Thus, AtCaM3 was used to study the interaction with AtPP7. The result showed that the yeast strain AH109 could not grow on the medium lacking Trp, Leu, His and adenine, when *AtPP7* was cotransformed with *AtCaM3* (Fig. 6a). However, the X-Gal filter assay showed positive reaction (Fig. 6b), indicating an instantaneous interaction between AtPP7 and AtCaM3. It was reported that the C-terminal region of PP7 contains multiple Ca^{2+} binding sites in humans (Huang & Honkanen 1998). Whether or not AtPP7 also has Ca^{2+} binding sites remains to be solved. If so, we assume that PP7 phosphatase may be controlled by Ca^{2+} in a dual way: via interaction with CaM or directly modulated by Ca^{2+}.

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SUPPLEMENTARY MATERIAL

The following supplementary material is available for this article:

Figure S1. Identification of the number of *AtPP7* gene insertion in the transgenic lines by Southern blot.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1365-3040.2006.01613x
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