Over-expression of heat shock protein gene *hsp26* in *Arabidopsis thaliana* enhances heat tolerance

Y. XUE[1], R. PENG[1], A. XIONG[1], X. LI[1], D. ZHA[2] and Q. YAO[1]*

Biotech Research Institute[1] and Horticultural Research Institute[2], Shanghai Academy of Agricultural Sciences, Shanghai, 201106, P.R. China

Abstract

In the yeast *Saccharomyces cerevisiae*, the molecular chaperone HSP26 has the remarkable ability to sense increases in temperature directly and can switch from an inactive to a chaperone-active state. In this report, we analyzed the effect of expression of HSP26 in *Arabidopsis thaliana* plants and their response to high temperature stress. The *hsp26* transgenic plants exhibited stronger growth than wild type plants at 45 °C for 16 h. The chlorophyll content and chlorophyll fluorescence decreased much more in wild type than in transgenic plants. Moreover, the transgenic plants had higher proline and soluble sugar contents, and lower relative electrical conductivity and malondialdehyde contents after high temperature stress. Furthermore, we found that over-expression of HSP26 in *Arabidopsis* increased the amount of free proline, elevated the expression of proline biosynthetic pathway genes and therefore enhanced *Arabidopsis* tolerance to heat stress.

Additional key words: high temperature stress, transgenic plants, yeast gene.

Introduction

Heat shock proteins (HSPs), functioning as molecular chaperones, can prevent the accumulation of protein precursors, accelerate the transport of proteins, and absorb complexes of unfolded proteins to maintain their transport abilities. HSPs can also sustain the normal folded state of proteins, degrade misfolded proteins, stabilize polypeptide strands, and prevent protein inactivity. In addition, HSPs participate in regulating the activation and function of target proteins, although they themselves are not components of the target proteins. HSPs with low molecular mass (about l5 to 30 kD) are called small heat shock proteins (sHSPs) (Sugiyama *et al*. 2000). Small heat shock protein family is a stress-inducible group of molecular chaperones that can prevent the polymerization of denatured protein. In the yeast *Saccharomyces cerevisiae*, the molecular chaperone HSP26 has the remarkable ability to directly sense increases in temperature and switch from an inactive to a chaperone-active state (Haslbeck 2002, Haslbeck *et al*. 2004, Franzmann *et al*. 2008). Like other sHSPs, HSP26 forms large oligomeric complexes. At heat shock temperatures, however, the 24mer chaperone complex dissociates. Interestingly, chaperone assays performed at different temperatures show that the dissociation of the HSP26 complex at heat shock temperatures is a prerequisite for efficient chaperone activity. Binding of non-native proteins to dissociated HSP26 produces large globular assemblies with a structure that appears to be completely reorganized relative to the original HSP26 oligomers. In this complex one monomer of substrate is bound per HSP26 dimer. The temperature-dependent dissociation of the large storage form of HSP26 into a smaller, active species and the subsequent re-association to a defined large chaperone-substrate complex represents a novel mechanism for the functional activation of a molecular chaperone.

Several studies correlated the induction of HSPs by mild heat stress with the induction of tolerance to much more severe stress (Ougham and Howarth 1988, Vierling 1991, Howarth and Skot 1994). In addition, over-expression of certain transcriptional regulators of HSP expression, HSF1 and HSF3, causes plants to constitutively express at least some HSPs and produces

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Abbreviations: EC - electrical conductivity; HSF - heat shock factor; HSP - heat shock protein(s); MDA - malondialdehyde; RT-PCR - reverse transcription - polymerase chain reaction; sHSP - small heat shock protein(s).

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* Corresponding author; fax: (+86) 21 52211055, e-mail: ebiosaas@gmail.com

somewhat higher basal heat tolerance (Lee *et al*. 1995, Prändl *et al*. 1998, Mishra *et al*. 2002). Some direct evidence exists for the function of an individual HSP in heat tolerance in plants. Changes in the expression of HSP17.7 after heat stress cause modest changes in growth rates of tissue culture cells and electrolyte leakage of leaves (Malik *et al*. 1999). Sanmiya *et al*. (2004) introduced the tomato *mt-sHSP* gene under the control of the 35S promoter into tobacco, and enhanced the heat tolerance in the transformed plants. Furthermore, *sHSP17.7* was overexpressed in the rice under the control of a CaMV 35S promoter and the transgenic rice plants exhibited significantly increased heat tolerance compared to untransformed plants (Murakami *et al*. 2004). However, information on heat tolerance mechanisms in these transgenic plants is limited.

To elucidate the possible role of *hsp26* gene in *Arabidopsis thaliana* in responses to heat stresses, we cloned *hsp26* gene from *Saccharomyces cerevisiae* through PCR method. Then, we transformed this gene into *Arabidopsis thaliana* and obtained results indicated that the *hsp26* gene was over-expressed in the transgenic plants after high temperature stress. Furthermore, we studied accumulation of defense proteins, free proline and soluble sugars in *Arabidopsis* under heat stress.

Materials and methods

Isolation of a cDNA encoding the putative HSP26 protein: The full cDNA was cloned by polymerase chain reaction (PCR) from the yeast *Saccharomyces cerevisiae* cDNA library. PCR products were cloned in the pUCm-T vector and sequenced by Chinese National Human Genome Center in Shanghai. One pair of special primers was used to get real full-length cDNA (the forward primer based on the start codon ATG: 5'-GGA TCC ATG TCA TTT AAC AGT CCA TTT TTT G-3' and the reverse primer based on the stop codon: 5'-GAG CTC TTA GTT ACC CCA CGA TTC TTG AG-3').

Transgenic plants over-expressing the *hsp26* gene: The *hsp26* gene was cloned into the binary vector pYG8401, which was constructed on the basis of the vector pCAMBIA-1301, with the hygromycin gene as the selection marker gene. The *hsp26* gene cassette, containing the double CaMV 35S promoter and Nos-Terminator in its downstream sequence, was flanked by two tobacco scaffold attachment regions (Fig. 1). The constructs were introduced into *Agrobacterium tumefaciens* GV3101 by electroporation. *Arabidopsis thaliana* was transformed by the floral dip method (Zhang *et al*. 2006).

Plant materials: Seeds of *Arabidopsis thaliana* L. were surface sterilized with bleaching powder (5 %, m/v) for 20 min, washed with sterile water three times, and placed in Petri dishes that contained Murashige and Skoog (1962; MS) medium with 0.8 % agar. The incubation and growth conditions for *A. thaliana* were as described by Zhang *et al*. (2006). Eight transgenic plants with *hsp26* were obtained through 50 μg cm^{-3} hygromycin selection, and the transgenic plants of T_2 generation were confirmed by GUS stain and PCR. For heat stress, the wild type and transgenic plants grown in pots (9 plants per each pot) were transferred to 45 ± 1 °C, relative humidity 70 % for 16 h and then returned to 22 °C for 1 d.

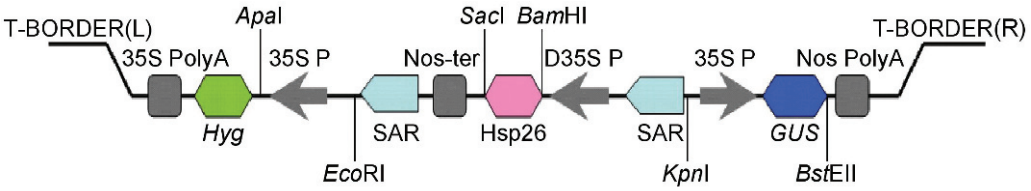

Fig. 1. Schematic diagram of the vector used in this study. The vector contains the double CaMV 35S (DCaMV35S) promoter and the tobacco mosaic virus (TMV) sequence fused to the *hsp26* gene. For steady transmission of the *hsp26* gene, two Scaffold attachment regions (SAR) were fused upstream of the DCaMV35S promoter and downstream of the Nos-terminator (Nos-ter).

Reverse transcription-PCR analysis: Total RNA was digested with DNase I (*Promega*, Madison, WI, USA) to remove genomic DNA. The first strand of cDNA was synthesized using 5 μg of total RNA as template with the reverse transcription system (*Promega*) in a 0.02 cm^3 reaction volume. In order to improve the reliability of RT-PCR, the *A. thaliana actin* gene (*AtAc2*, accession number NM112764) was synthesized by two primers (*AtAc2*Z1: 5'-GCA CCC TGT TCT TCT TAC CGA G-3'; *AtAc2*F1: 5'-AGT AAG GTC ACG TCC AGC AAG G-3') and was used as an internal standard. The PCR reaction was carried out in 27 cycles of 40 s at 94 °C, 30 s at 58 °C, and 20 s at 72 °C, plus a final extension at 72 °C for 5 min. A 190-bp fragment of the *hsp26* gene was amplified using two specific primers (*hsp26F*1: 5'-AAC AGA TTG CTG GGT GAA GG-3'; *hsp26R*1: 5'-AAA CCA GAT GGG AAC AGG GA -3') according to the sequence of the *hsp26* gene and similar PCR reaction conditions as *AtAc2*. The PCR products were separated on 2 % agarose gel and quantified using a *Model Gel Doc 1000* (*Bio-Rad*, Hercules, USA). DNA of the *hsp26* gene to *AtAc2* ratio was analyzed with *ShineTech Gel Analyser* (*Shine Science*

of Technology Co., Shanghai, China) to evaluate the expression pattern of the *hsp26* gene. The same results were obtained for three independent experiments, therefore only the result from one experiment is presented.

Determination of chlorophyll content and chlorophyll fluorescence: Chlorophyll was extracted from individual leaves using 95 % ethanol. Chlorophyll contents were determined according to Lichtenthaler (1987).

Photochemical efficiency of photosystem 2 (PS 2) was determined by variable to maximum chlorophyll fluorescence ratio (F_v/F_m) measured on the upper surface of the leaves with a *Plant Efficiency Analyser* (*PAM-2100*, Walz, Effeltrich, Germany) according to Tarantino *et al*. (1999). Before measurements, leaves were dark-adapted for 30 min at room temperature.

Measurements of relative electrical conductivity and lipid peroxidation: Samples were separated into two equal groups. The first group was shaken in 5 cm^3 double-distilled water at 170 rpm for 2 h at 25 °C, the second group was boiled for 30 min. The relative electrical conductivity (EC) was measured as described by Tang (1999). The relative conductivity [%] was calculated as the ratio of EC of intact leaves and EC of boiled leaves × 100.

The lipid peroxidation in leaves was estimated by measuring the amount of malondialdehyde (MDA), a decomposition product of the oxidation of polyunsaturated fatty acids, as described by Havaux *et al*. (2003).

Measurements of soluble sugars and proline: Soluble sugars were extracted from the frozen leaves in 90 % ethanol and quantified using a phenol-sulfuric acid assay with sucrose as a standard (Farrar 1993). Proline content was measured in acidic extracts and quantified spectrophotometrically using acid-ninhydrin reagent with proline as a standard (Lea and Blackwell 1993). We repeated the sugar and proline measurements three to five times on independent batches of plant material.

Real-time reverse transcription-PCR analysis: Amplification of specific regions of targeted genes were carried out using the *iQ SYBR Green Supermix* kit (*BioRad*), and real-time detection of production was performed in a *Mini Opticon* real time PCR system (*BioRad*). The real-time PCR primer sequences were designed using Primer 3 software, and the primer sequences were listed in Table 1. All cDNA samples were analyzed in duplicate or triplicate, and the cDNA was derived from two sets of independently grown plants. Thermal cycling conditions consisted of 38 cycles of 94 ºC for 20 s, 58 ºC for 30 s, 72 ºC for 20 s, and a final extension at 72 ºC for 5 min. The relative changes in gene expression were quantified according to Livak and Schmittgen (2001).

Table 1. Real-time PCR primers used for analyzing the expression of genes.

Gene name	Primer sequences (5' to 3')
AtP5CS1	CACAACGCCAGCACAAGATTC and AGCTTGGATGGGAATGTCCTG
AtP5CS2	GGTTTTAGGTTCGGACTTGGTG and AATTCCATTCTCAACAGCCTC
AtP5CR	AGTTTAGCTTCACAGACCGTTC and GCTCTGTGAGAGCTCGCGGCTTC
AtProDH1	GGTATGTCAGATGCATTGTCC and CGCAATCCCGGCGATTAATCTC
AtProDH2	GGGAAGATCGAGTTTGCGCAG and CCAAGCCATAACTCTTCTCTTAAG
AtOAT	GATCCGGCATTTGCACCTGGAG and TTTCGAGATTTTCGCAGCTAATTC

Results

Through selection, we obtained transgenic plants that were identified by RT-PCR (Fig. 2*A*). We selected four transgenic lines (named T1, T2, T3 and T4, corresponding to lanes 2, 3, 4, and 5 in Fig. 2*A*, respectively) that demonstrated relatively high expression of *hsp26*. All transgenic plants survived heat shock (45 °C for 16 h) while 78 % of the wild type plants died (Fig. 2*B*).

The total chlorophyll content in wild type and three selected transgenic lines plants (T1, T2, and T3) was remarkably decreased after heat stress and recovery. However, the decrease in the transgenic plants was significantly lower than that in wild type plants (Table 2). Wild type and transgenic plants were exposed to high irradiance of 400 μmol m^{-2} s^{-1} and high temperature of 45 °C for 16 h. And after dark adaptation for 30 min F_v/F_m was measured at room temperature. High temperature treatment caused a higher decrease in the F_v/F_m values, which indicated more severe damage of PS 2 in wild type plants than in transgenic plants (Table 2). This suggested that plants over-expressing the *hsp26* gene were more tolerant to photoinhibition than wild type plants.

The relative electrical conductivity of transgenic plants was significantly lower than that of wild types after high temperature stress (Table 2), demonstrating lower membrane damage in transgenic plant. Higher MDA content was detected in wild type than in transgenic plants especially after heat stress. The transgenic plant had higher proline content at normal conditions and the increase after heat stress was significantly higher than in transgenic plants that in wild type plants (Table 2). After high temperature stress, also the content of soluble sugars was significantly higher in transgenic than in wild plants.

Table 2. Effects of high temperature (45 °C, 16 h) on the physiological properties in leaves of wild type and transgenic plants (T1 to T3). Data are the means ± SD of three replicates.

		WT	T1	T2	T3
Chlorophyll content [mg g^{-1}(f.m.)]	control	1.76 ± 0.10	1.65 ± 0.09	1.83 ± 0.12	1.82 ± 0.11
	heat	0.78 ± 0.05	1.10 ± 0.06	1.25 ± 0.07	1.25 ± 0.06
F_v/F_m	control	73.23 ± 1.66	70.64 ± 1.52	75.24 ± 1.75	76.35 ± 1.79
	heat	12.42 ± 0.93	30.85 ± 0.82	36.75 ± 1.33	38.27 ± 1.22
Relative electrical conductivity [%]	control	2.13 ± 0.33	2.05 ± 0.26	1.94 ± 0.21	1.65 ± 0.23
	heat	29.67 ± 0.68	15.24 ± 0.48	10.35 ± 0.38	9.86 ± 0.47
MDA content [µmol g^{-1}(f.m.)]	control	6.28 ± 0.42	6.17 ± 0.21	6.05 ± 0.37	5.71 ± 0.40
	heat	10.99 ± 0.58	9.15 ± 0.47	9.05 ± 0.50	8.62 ± 0.53
Proline content [µmol g^{-1}(f.m.)]	control	0.26 ± 0.04	0.90 ± 0.04	1.06 ± 0.15	1.06 ± 0.13
	heat	2.48 ± 0.05	8.52 ± 0.13	11.95 ± 0.23	11.99 ± 0.19
Sugar content [µmol g^{-1}(f.m.)]	control	6.06 ± 0.12	6.08 ± 0.14	6.21 ± 0.15	6.18 ± 0.14
	heat	8.56 ± 0.18	11.46 ± 0.18	14.08 ± 0.21	13.98 ± 022

Fig. 2. *A* - confirmation of *hsp26* genomic integration in transgenic lines by RT-PCR. Wild type (WT) and transgenic lines (T1 - T4) of *A. thaliana* were used as PCR templates. Specific primer pairs for PCR of *hsp26* were used. *B* - Thermotolerance of the D35S: *hsp26* transgenic *A. thaliana*. Five-week-old wild type and transgenic plants grown in normal conditions (*upper panels*) and after high temperature treatment (45 ℃, 36 h) (*lower panels*).

Table 3. Relative transcription level of proline biosynthetic and degradation pathways genes in wild type and transgenic plants (T1 to T3) during non-heat and heat treatment. Total RNA was isolated, converted into cDNA, and subjected to quantitative RT-PCR assays. Relative amounts were calculated and normalized with respect to the expression of each gene in WT without heat treatment. Values were the means ± SD of three assays.

Genes		WT	T1	T2	T3
AtP5CS1	control	1.00 ± 0.17	1.92 ± 0.26	2.19 ± 0.25	2.01 ± 0.24
	heat	5.63 ± 0.47	9.24 ± 0.65	10.54 ± 0.55	9.82 ± 0.45
AtP5CS2	control	1.00 ± 0.27	2.12 ± 0.36	2.49 ± 0.35	2.22 ± 0.34
	heat	6.42 ± 0.48	10.18 ± 0.67	11.53 ± 0.57	9.78 ± 0.54
AtP5CR	control	1.00 ± 0.18	1.02 ± 0.24	1.19 ± 0.24	0.98 ± 0.21
	heat	4.63 ± 0.38	6.24 ± 0.45	6.53 ± 0.55	6.33 ± 0.44
AtProDH1	control	1.00 ± 0.27	1.05 ± 0.16	1.18 ± 0.44	0.85 ± 0.35
	heat	7.62 ± 0.37	10.21 ± 0.52	9.81 ± 0.42	10.81 ± 0.46
AtP5DH2	control	1.00 ± 0.15	0.96 ± 0.21	1.09 ± 0.21	1.01 ± 0.20
	heat	6.64 ± 0.57	9.73 ± 0.52	10.27 ± 0.54	10.77 ± 0.55
AtOAT	control	1.00 ± 0.19	1.02 ± 0.29	1.12 ± 0.29	1.05 ± 0.30
	heat	4.59 ± 0.37	6.18 ± 0.36	6.48 ± 0.45	7.19 ± 0.54

To further elucidate the role of *hsp26* gene in response to heat stress, we analyzed the expression of proline biosynthetic and degradation pathways genes in transgenic and wild type plants. These genes included *AtP5CS1, AtP5CS2, AtP5CR*, *AtProDH1, AtProDH2* and *AtOAT*. There was no significant difference in the expression levels of *AtP5CR*, *AtProDH1, AtProDH2* and *AtOAT* between non-treated transgenic lines and wild type plants. However the *AtP5CS1* and *AtP5CS2* mRNAs showed high accumulation in transgenic lines, compared to wild type plants under normal conditions (Table 3). After heat stress, the expression of all six proline biosynthetic pathway genes in transgenic lines was increased when compared to wild type plants (Table 3).

Discussion

In this paper, we studied yeast *hsp26* gene function through transferring this gene into *Arabidopsis thaliana*. We found that high temperature treatment caused significant declines in chlorophyll content and F_v/F_m in both wild type and transgenic lines (Table 2). Moreover, chlorophyll content and F_v/F_m were lower in wild type compared to transgenic lines after heat stress. Chlorophyll fluorescence analysis has become one of the most powerful and widely used techniques for evaluation of the effects of environmental stresses (Maxwell and Johnson 2000). Zoran *et al*. (2007) studied the relationship between the heat stability of thylakoids and loss of chlorophyll in winter wheat (*Triticum aestivum* L.) under heat stress. Our findings are consistent with their results that heat stress caused a significant decline in chlorophyll content. On the other hand, the protection of PS 2 electron transport by the chloroplast sHSP during heat stress has been demonstrated by an *in vitro* experiment (Heckathorn *et al*. 1998), though its mechanism remains unsolved. Therefore, it is possible that the sHSP plays an important role in protecting the photosynthetic machinery, especially PS 2, against damage caused by heat stress.

The accumulation of MDA is often used as an indicator of lipid peroxidation (Smirnoff 1995). High temperature stress increased MDA content both in wild type and transgenic lines (Table 2), similar to what has been found in other species (*e.g*. Gong *et al*. 1997). Less MDA accumulation in transgenic lines than in wild type plants and low relative electrical conductivity suggested a lower membrane damage in transgenic plants at high temperature. This implies that the HSP26 protein could protect the cell membrane integrity of *Arabidopsis* plant under heat stress. Previously, HSP synthesis was found to preserve plasma membrane structures and diminish solute leakage (Lin *et al*. 1985). The high temperature tolerance conferred by HSPs has been partially attributed to the association of HSPs with membranes (Mansfield *et al*. 1988). Further experiments are needed to clarify the relationship between HSP26 and membranes, and its role in the maintenance of normal membrane structure during high temperature stress.

Proline and soluble sugar accumulation are another important factor in determining heat tolerance that can have a major role in osmotic adjustment (Voetberg and Sharp 1991, Wahid and Close 2007) and may also have a number of other protective roles. It was shown in transgenic tobacco plants that over-expression of proline in plants may lead to increased tolerance against these abiotic stresses (Kavi *et al*. 1995). In this experiment, transgenic plants showed normal growth after stress. The level of heat tolerance and the accumulation of proline and soluble sugars parallel one another after heat stress, indicating that proline and sugar accumulation are a fundamental component of enhanced heat tolerance. We

reasoned that if the increase in proline and soluble sugar that occurs in *Arabidopsis* with high temperature was brought about by genes that were regulated by the *hsp26*, then over-expression of HSP26 might result in elevated contents of proline and soluble sugar in plants. Further investigation should be done to elucidate more about the function and the regulatory mechanism of HSP26 in plant-stress responses.

It should be emphasized that proline content in transgenic plants was higher than in wild type plants also under non-treated conditions. Furthermore, real-time qPCR analysis revealed that the expressions of *AtP5CS1* and *AtP5CS2* encoding proline synthetase in transgenic plants were higher than that in wild type plants under both normal and heat stress conditions (Table 3). These findings suggest that enhanced stress tolerance of transgenic plants might occur partially due to HSP26-mediated activation of proline synthesis. However, we did not find out that over-expression of HSP26 in *Arabidopsis* could result in enhanced expression of other biosynthetic (*AtP5C*R, *AtOAT*), and degradation (*AtProDH1, AtProDH2*) pathways genes in transgenic plants under normal conditions (Table 3). *AtP5CS1*, *AtP5CS2, AtP5CR and AtOAT* genes were reported to increase under abiotic stress in *Arabidopsis* (Verbruggen *et al*. 1996, Strizhov *et al*. 1997). *AtProDH* played a key role in proline degradation in *Arabidopsis* (Mani *et al*. 2002), and was upregulated by proline (Kiyosue *et al*. 1996). These studies corroborated our results that the expression levels of all proline biosynthetic pathway genes were increased during stress. Under heat stress treatments, the expression levels of all proline biosynthetic pathway genes in HSP26 transgenic lines were increased more than that in wild type plants. One possible explanation for this observation is that HSP26 mediated the activation of such proline biosynthetic pathway genes accompanied with other stress-responsive regulator(s). Alternatively, there are stress negative regulator(s) with the role in repressing these stress-related genes in *Arabidopsis* under normal conditions. The high level of negative regulator(s) may repress expression of proline biosynthetic pathway genes activated by HSP26 in transgenic plants under normal conditions, while these transcripts would be significantly accumulated in transgenic plants when negative regulators are down-regulated by abiotic stresses.

Taken together, our results indicate that over-expression of HSP26 in *Arabidopsis* may contribute to the accumulation of defense proteins, as well as free proline and soluble sugars that function as stress protectors, by regulating expression of stress-related genes in *Arabidopsis* under heat stress conditions. Further investigation should be done to gain more information about the function and the regulatory mechanism of HSP26 in plant-stress responses.

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