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The involvement of chloroplast HSP100/ClpB in the acquired thermotolerance in tomato

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Abstract The chloroplast HSP100/ClpB is a newly documented member of the ClpB family, but little was known about its role in imparting thermotolerance to cells. A cDNA coding for a HSP100/ClpB homolog has been cloned from *Lycopersicon esculentum* and termed as *Lehsp100/ClpB* (the cDNA sequence of *Lehsp100/ClpB* has been submitted to the GenBank database under accession number: AB219939). The protein encoded by the cDNA was most similar to the putative chloroplast HSP100/ClpBs in higher plants and the ClpB from *Cyanobacterium Synechococcus* sp. A 97 kDa protein, which matched the predicted size of mature LeHSP100/ClpB, was immunologically detected in chloroplast isolated from heat-treated tomato plants. In addition, the fusion protein, combining the transit sequence of LeHSP100/ClpB and GFP, was found to be located in chloroplast based on the observations of fluorescent microscope images. These results indicated the chloroplast-localization of LeHSP100/ClpB. Both the transcript and the protein of *Lehsp100/ClpB* were not detected under normal growth conditions, but they were induced by increasingly higher temperatures. An antisense *Lehsp100/ClpB* cDNA fragment was introduced into the tomato by Agrobacterium-mediated transformation. Antisense lines exhibited an extreme repression of heat-induced expression of *Lehsp100/ClpB*. The levels of chloroplast HSP60 and small HSP in antisense lines were identical to those of the control plants. After plants preconditioned at 38°C for 2 h were exposed to a lethal heat shock at 46°C for 2 h, the antisense lines were greatly impaired and withered in 21 days of the recovery phase, whereas the untransformed control plants and the vector-transformed plants survived. Furthermore, chlorophyll fluorescence measurements showed that PS II in antisense lines were more susceptible to the thermal irreversible inactivation than the untransformed and vector-transformed control plants. This work provides the first example that induction of chloroplast LeHSP100/ClpB contributes to the acquisition of thermotolerance in higher plants.

Keywords Acquired thermotolerance · Chloroplast · HSP100/ClpB · Heat shock · Transgenic tomato

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Introduction

An elevation in temperature triggers a stress response found in all organisms. One of the main aspects of the heat shock response is the synthesis of heat shock proteins (HSPs). HSPs can be classified into five groups on the basis of their molecular masses: HSP100, 90, 70, 60, and small molecular weight HSPs (smHSPs). HSP100 proteins (also known as Clp [caseinolytic protease proteins]) are a class of diverse molecular chaperones involved in a wide variety of essential metabolic processes throughout prokaryotes and eukaryotes (Schirmer et al. 1996). In principle, two types of Clp/HSP100 proteins are distinguished. The members of Class 1, such as ClpA and ClpB, possess two distinct but conserved NBDs (Nucleotide Binding Domains), whereas the members of class 2, such as ClpX and ClpY (HslU), have a single NBD (Sauer

et al. 2004). Members of the ClpB family differ from other Clp/HSP100 proteins by possessing a longer middle region (M-domain). ClpA and ClpX function as ATPases to regulate ClpP-mediated proteolysis (Hoskins et al. 1998; Singh et al. 2000), but ClpB family members possess molecular chaperone activities to rescue stress-damaged proteins from an aggregated state.

The HSP100 is conserved in bacteria, yeast, and plants (also known as ClpB, HSP104, and HSP101, respectively). HSP100/ClpB proteins play an essential role in thermotolerance in many organisms. Loss of ClpB synthesis in *E. coli* significantly reduces cell viability after a sudden and extreme heat shock, but does not affect the cell's ability to develop thermotolerance (Squires et al. 1991). The best-studied *ClpB* gene is yeast HSP104. Deletion of *hsp104* in *Saccharomyces cerevisiae* led to the loss of acquired thermotolerance and greatly reduced the ability of cells to survive high-temperature exposure (Sanchez and Lindquist 1990).

The plant HSP100/ClpB family is classified into a few subfamilies according to phylogenetic relationships or their subcellular localization (Agarwal et al. 2001). The plant HSP101 subfamily is a cytosolic or nuclear member of the ClpB protein family. Plant *hsp101* genes have been isolated from *Arabidopsis* (Schirmer et al. 1994), soybean (Lee et al. 1994), tobacco (Wells et al. 1998), rice (Agarwal et al. 2003), maize (Nieto-Sotelo et al. 1999) and wheat (Wells et al. 1998). Plant HSP101s share a great similarity with yeast HSP104 (Gallie et al. 2002) and can complement a thermotolerance defect in yeast caused by the mutation of the *hsp104* gene (Lee et al. 1994; Schirmer et al. 1994; Wells et al. 1998), suggesting the similarity of their molecular chaperone activities. The genetic analysis of mutant *hot1* (sensitive to hot temperatures) indicated that the acquisition of thermotolerance in *Arabidopsis* was related to the loci of *hsp101* genes (Hong and Vierling 2000). The inhibition of *hsp101* expression by antisense RNA severely compromises thermotolerance of *Arabidopsis* seedlings (Queitsch et al. 2000). Overexpression of rice HSP101, which complemented the acquired thermotolarance defect in yeast *hsp104* mutant, enhanced the basal thermotolerance in transgenic rice (Agarwal et al. 2003). Plenty of experiment data indicated that plant HSP101 plays an important role in acquired thermotolerance.

Keeler et al. (2000) cloned a cp *hsp100/ClpB* gene from lima bean (*Phaseolus lunatus*) and the corresponding protein is putatively located in chloroplast. The accumulation of cp HSP100/ClpB in lima bean was tightly correlated with heat acclimation, suggesting that the chloroplast HSP100/ClpB may be one of the important proteins for acquired thermotolerance. Even though bioinformation investigation revealed the ubiquity of chloroplast HSP100/ClpB homolog, little research was conducted for this gene family, except for the primary study on lima bean cp HSP100/ClpB (Keeler et al. 2000).

In the present study, we described the isolation of the *Lehsp100/ClpB* gene of tomato and reported its involvement in the acquired thermotolerance. These results suggest that LeHSP100/ClpB plays an important role in the thermotolerance of tomato plants.

Materials and methods

Plant materials and heat stress

Lycopersicon esculentum cv. Zhongshu 4 plants were grown at a 30/25°C (day/night) temperature regime, under natural (540 μmol m^{-2} s^{-1}) illumination. The 6-week-old tomato plants were used for extraction of RNA and evaluation of thermotolerance. All heat shock treatments were performed under natural (540 μmol m^{-2} s^{-1}) illumination. The recovery of the stressed plants was under normal growth condition.

Cloning of tomato *Lehsp100/ClpB* cDNA

Total RNA was isolated from the leaves of tomato plants heat-shocked at 39°C for 2 h. The mRNA was isolated from total RNA using an oligo(dT)-cellulose chromatography column. A cDNA library in lambda ZAP-II was constructed following the instruction manual of Stratagene ZAP-II cDNA Synthesis. A cDNA library with a titration of 1.5×10^6 phages/μg λ DNA was obtained, 1000 clones of which were selected randomly for sequencing. The GenBank program BlastX was used to search similar genes and proteins. A phylogenetic tree was generated by the maximum parsimony method (PAUP software, version 4.0b4a).

Determination of the intracellular location of LeHSP100/ClpB

After wild type tomato plants were treated at 38°C for 2 h, the intact chloroplasts were isolated as described previously (Bruce et al. 1994). The isolated chloroplasts were subjected to Western-blotting analysis to verify the predicted chloroplast-localization of LeHSP100/ClpB.

As an alternative, a GFP fusion was used to determine where the LeHSP100/ClpB was located *in vivo*. A cDNA fragment, encoding the first 80 amino acids at

the N-terminal of LeHSP100/ClpB, was amplified by PCR using the primers 5′-AATTAACCCTCACTAAAGGG-3′ and 5′-CTATCTCGAGCGATGCAACAATTGC-3′. The PCR-amplified fragments were digested with *Bam*H I and *Xho* I restrictive enzymes, and then inserted into the corresponding sites of a p35S-sGFP vector. The constructed vector was introduced into the tobacco following the method described by Jin et al. (2001). By means of confocal laser scanning microscopy, the transgenic tobacco cell was illuminated with blue light (488 nm) that causes GFP fusion and chlorophyll to fluoresce green and red, respectively. By means of filter (660 nm) or filter (505–530 nm), the red and the green fluorescences were collected, respectively. The merged image of the red and the green fluorescences was exported for the distribution analysis of the GFP fusion.

RNA extraction and Northern blot analysis

Total RNA was extracted using the guanidine thiocyanate method described by Ragueh et al. (1989). A 30 μg of total RNA were separated on denaturing formaldehyde agarose gels (1.5%) and blotted onto a Hybond-N membrane. The first 795 bp of *Lehsp100/ClpB* cDNA was used as a probe, which was labeled α-^{32}P-dCTP. Hybridization was carried out at 65°C in hybridization solution (0.5 M sodium phosphate, 7% (W/V) SDS, 10 mM EDTA, and 100 μg/ml salmon sperm DNA). After hybridization, membranes were washed with wash solution (2 × SSC, 0.1% SDS for 15 min at 60°C and with 0.1 × SSC, 0.1% SDS for 10 min at 60°C), and hybridization signals were visualized via autoradiography.

Production of polyclonal antibody against LeHSP100/ClpB

The fusion protein, combining GST and the first 140 amino acids of LeHSP100/ClpB, was expressed in *E. coli* with reconstructed pGEX-6P-1 vector, and then purified through the glutathione–agarose affinity chromatography. The purified fusion protein was injected in New Zealand white rabbits to generate the polyclonal antibody.

Western-blot analysis

From the leaves of 6-week-old tomato plants, total protein was extracted in the extraction buffer (1 mM PMSF, 10 mM Tris–HCl, and pH 8.0). Protein concentration was primarily determined by using a Coomassie Blue dye-binding assay with BSA as the standard and was further accurately quantified by A.A.B Image Analysis Software according to SDS-PAGE gel image. Equivalent total protein was loaded on SDS-PAGE gel for Western analysis. The anti-HSP100/ClpB antibody was used at a dilution of 1:50 in TBS (2% Tween-20, 20 mM Tris–HCl, pH 7.5, and 500 mM NaCl).

Construction of *Lehsp100/ClpB* antisense expression vector and tomato transformation

The first 330 bp *of Lehsp100/ClpB* cDNA was amplified by PCR with the primers: 5′-AATTAACCCTCACTAAAGGG-3′ and 5′-TCTGGATCCGCAACAATTGCTTG-3′. The PCR products were digested with *Bam*H I and then ligated into pBlueScript (SK) vector in the forward orientation, thus generating pBS-1 vector. Another DNA fragment, corresponding to the 3021–3144 bp of *Lehsp100/ClpB* cDNA, was amplified by PCR with the following primers: 5′-TAGTAGTAGCTCTTGTATCT-3′ and 5′-TAAGAGCTCTCACTATAGGGC-3′. The PCR products were digested with *Sac* I and *Xba* I, and then inserted into the pBS-1 vector, resulting in a pBS-2 vector. The pBS-2 vector was digested with *Sac* I and *Kpn* I, and the released fragment was inserted into pROK II, finally generating the antisense *Lehsp100/ClpB* vector.

The constructed vectors were introduced into *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method (Holsters et al. 1978). Tomato leaf disk transformation was performed according to Horsch et al. (1985) method.

Chlorophyll fluorescence emission determination

The photochemical efficiency of PSII was monitored by means of chlorophyll fluorescence measured by a Plant Efficiency Analyzer (FMS2, Hansatech Instruments Ltd., Norfolk, UK). Measurements were taken on the upper surface of leaves, which had been darkened for at least 30 min after various treatments. The measured chlorophyll fluorescence data included: initial fluorescence (F_o) upon exposure to weak light after dark adaptation, maximum fluorescence yield (F_m) of a light adapted leaf exposed to a pulse of saturating light. The potential maximal efficiency of PSII (F_v/F_m) of dark-adapted leaves was calculated as $F_v/F_m = (F_m - F_o)/F_m$.

In the PS II thermotolerance experiment, plants grown under normal conditions were subjected to successive heat treatments, in steps of every 2°C, from 38 to 46°C. At each temperature stage, heat stress was retained for 2 h. The light density during heat stress was 540 μmol m^{-2} s^{-1}. After heat treatments, plants

were returned to normal conditions for recovery. The chlorophyll fluorescence was measured after 30 min of dark period.

Results

Cloning of *Lehsp100/ClpB*

From the 1000 EST data, a 2130 bp-long cDNA clone showed a significant similarity to the known lima bean cp *hsp100/ClpB* gene through a BlastX search. This cDNA was then used to further screen the cDNA library for full-length clones of putative chloroplast *hsp100/ClpB*. The longest clone isolated is 3144 bp, containing an ORF of 980 amino acids and corresponding to a predicted polypeptide of 110.3 kDa. The identified clone was therefore termed *Lehsp100/ClpB*.

The transcript or the protein of *Lehsp100/ClpB* was not detected at the normal growth temperature of 28°C. *Lehsp100/ClpB* gene was induced by evaluated temperatures and the accumulations of both its transcript and its protein reached a peak at 38°C (Fig. 1).

Chloroplast-localization of LeHSP100/ClpB

The predictions of on-line program ChloroP and TargetP revealed 76 aa of chloroplast-targeted transit sequence in LeHSP100/ClpB. The predicted chloroplast-localization was primarily confirmed by immunodetection of LeHSP100/ClpB in purified chloroplast fraction (Fig. 2). As an alternative, chloroplast-localization was corroborated based on the microscope

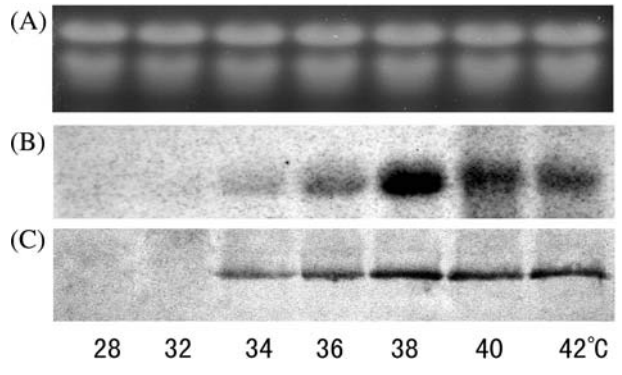

Fig. 1 Heat stress-inducible expression of *Lehsp100/ClpB* in tomato leaves. The 6-week-old plants grown under normal condition were heat shocked at the indicated temperatures for 2 h. Ribosomal RNA was used as a control for equilibrium loading (**A**). Northern blots were probed with the first 795 bp fragment of *Lehsp100/ClpB* cDNA (**B**). The chloroplast proteins were fractionated by SDS-PAGE and immunoblotted using an anti-LeHSP100/ClpB serum (**C**)

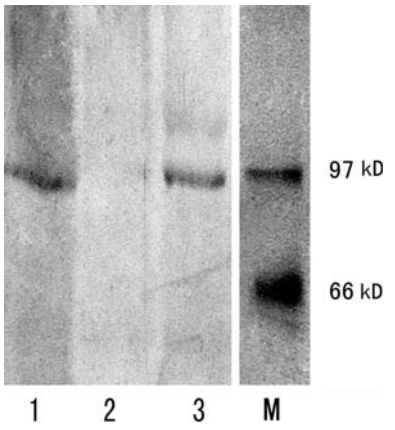

Fig. 2 Western-blot analysis of LeHSP100/ClpB in purified chloroplast. The proteins were extracted, respectively, from leaves treated at 38°C for 2 h (lane 1), the chloroplasts purified from normal tomato leaves grown at 28°C (lane 2) and the chloroplasts purified from the tomato leaves treated at 38°C for 2 h (lane 3). The extracted proteins were separated by 8% SDS-PAGE and then subjected to Western-blot (lane 1–3). Lane M presents protein markers

images of GFP fusion protein that consisted of both lead sequence (transit sequence of LeHSP100/ClpB) and GFP (Fig. 3A). The stomatal cells on leave epidermis peeled from transgenic tobacco were illuminated with blue light (488 nm) that caused GFP and chlorophyll to fluoresce green and red, respectively. The mergence of the two color fluorescent images revealed a co-localization of GFP with the red signal of autofluorescent chlorophyll (Fig. 3B). All these results indicated undoubtedly that LeHSP100/ClpB was a chloroplast protein.

Phylogenetic relationship of HSP100/ClpB

Figure 4 showed the phylogenetic relationship of 17 full-length HSP100/ClpB proteins from many organisms. The cytosolic/nuclear HSP101s and putative chloroplast HSP100/ClpBs were separately clustered into disjunct bushes. LeHSP100/ClpB and four putative chloroplast HSP100/ClpBs were included in bush I. LeHSP100/ClpB was most homologous to an *Arabidopsis* HSP100/ClpB (At2g25140) and largely to rice HSP100/ClpB (AAS07199). Lima bean cp HSP100/ClpB (AAF91178) and another putative chloroplast HSP100/ClpB from *Arabidopsis* (At2g25140) occurred in the adjacent subcluster. All putative chloroplast HSP100/ClpB were closely related to *Cyanobacterium Synechococcus* ClpB (AAB09631), implying the endosymbiont origin of plant chloroplast HSP100/ClpB.

The well-studied plant cytosolic/nuclear HSP101s were clustered together in bush II. One HSP101-like protein from the moss *Funaria hygrometrica* was

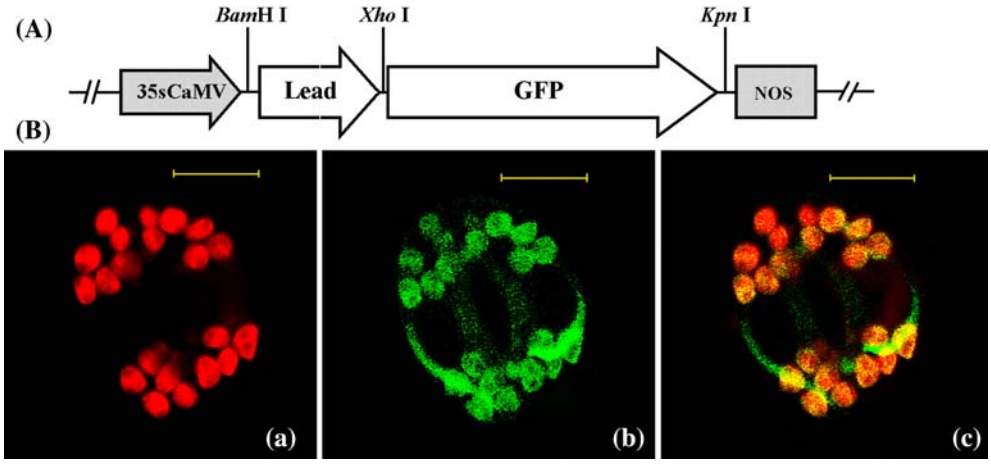

Fig. 3 Confocal laser scanning micrographs of tobacco leaf stoma cells stably expressing the GFP fusion protein. (**A**) Expression vector of GFP fusion protein consisting of lead sequence (transit sequence of LeHSP100/ClpB) and GFP. (**B**): (**a**) The autofluorescence of chloroplast in stomatal cell. (**b**) The fluorescence of GFP in stomatal cell. (**c**) Superposition of green fluorescence (GFP) and red fluorescence (chlorophyll) in stomatal cell. The length of the bar corresponds to 10 μm

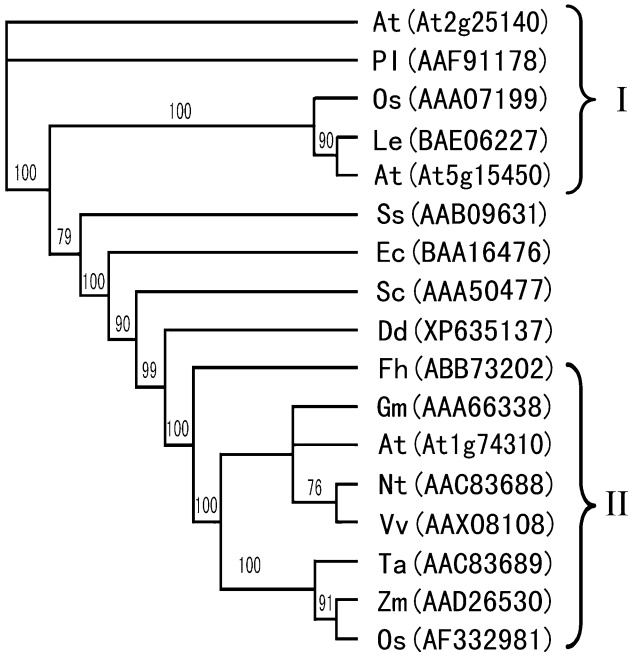

Fig. 4 Phylogenetic analysis of HSP100/ClpB sequences. The tree presented here was constructed by the parsimony method with the PAUP program. The numbers above the branches are the calculated bootstrap values. The organisms in the present phylogenetic tree include *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Escherichia coli* (Ec), *Saccharomyces cerevisiae* (Sc), *Synechococcus* sp. (Ss), *Vitis vinifera* (Vv), *Glycine max* (Gm), *Lycopersicon esculentum* (Le), *Phaseolus lunatus* (Pl), *Nicotiana tabacum* (Nt), *Triticum aestivum* (Ta), *Zea mays* (Zm), *Dictyostelium discoideum* (Dd), and *Funaria hygrometrica* (Fh). The accession numbers in GenBank of the proteins are noted beside the abbreviations of species

noticeably present in this bush. Some well-studied bacterium ClpB, including yeast HSP104 (AAA59477), *E. coli* ClpB (BAA16476) and the only one ClpB-like gene (XP635137) in *D. discoideum* genome, were clustered between the bushes I and II in the phylogenetic tree. This relationship was also supported by the Blast search result, which indicated that plant chloroplast HSP100/ClpBs and cytosolic/nuclear HSP101s were more closely related to bacterium ClpBs other than their each other. The evolutional divergence between chloroplast and cytosolic/nuclear ClpBs could be traced back to early stage of evolutional history.

The repression of LeHSP100/ClpB expression in antisense lines

In order to specifically repress the expression of *Le-hsp100/ClpB*, the highly variable region, containing 5′-UTR and the following coding region for chloroplast transit sequence of LeHSP100/ClpB, was used for the construction of the antisense vector. Furthermore, 312 bp of 3′-UTR, including a ploy-A tailing signal, was attached to the antisense fragment in order to increase the stability of the antisense mRNA and to enhance the efficiency of repression. According to this strategy, the antisense vector was constructed on the base of the pROK II vector (Fig. 5A)

In the present study, we generated 13 antisense transformants, seven of which produced homozygotic lines. As expected, the expression of LeHSP100/ClpB diminished so severely as to be undetectable in three lines (AS1, 4 and 7) (Fig. 5B, C). These three lines were selected for further evaluation of thermotolerance.

We also checked the expression of other HSPs. The constitutive protein levels of HSC70 or chloroplast HSP60 in antisense lines were found to be comparable to untransformed plants (Fig. 6). This indicated not

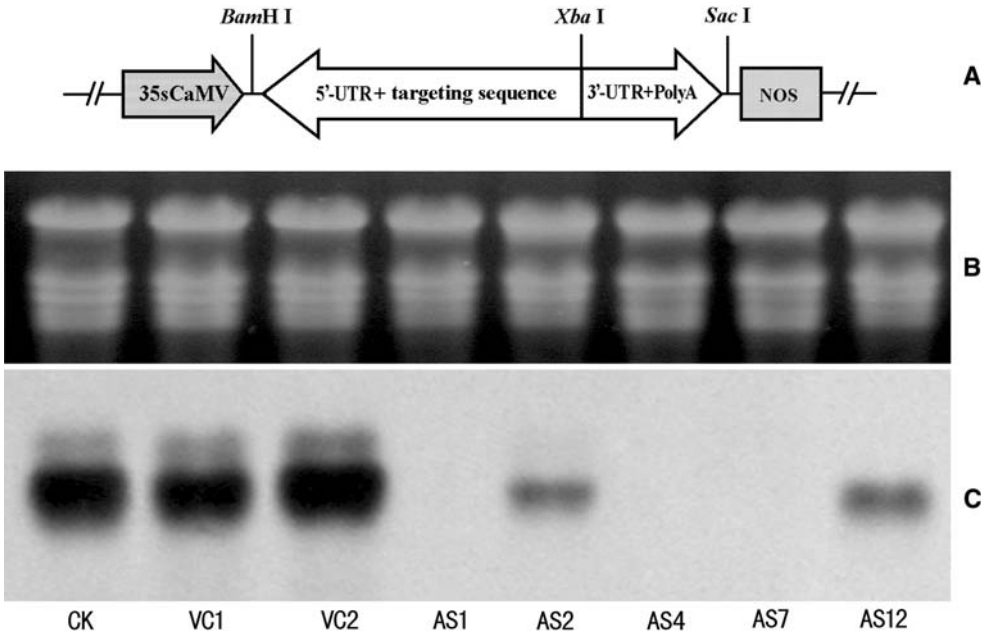

Fig. 5 Repression of LeHSP100/ClpB expression in antisense transgenic lines. Antisense vector of *Lehsp100/ClpB* (**A**) was introduced into tomato to generate antisense transgenic plants. Total RNA was extracted from the antisense transgenic (AS), pROK II-transformed control (VC), and untransformed plants (CK), all of which had been treated by a high temperature at 38°C for 2 h. Ribosomal RNA was used as a control for equilibrium loading (**B**). Northern Blots were probed with the first 795 bp of *Lehsp100/ClpB* cDNA (**C**)

only that constitutive expressions of these HSPs were not disturbed in antisense lines, but also that an equivalent amount of protein was loaded for Western-blot analysis. Furthermore, the protein levels of chloroplast small HSP and chloroplast HSP60 in antisense lines were nearly identical to those in untransformed plants and pROK II-transformed control under the heat shock conditions (Fig. 6). Therefore, a decrease in both transcript and protein levels of *Lehsp100/ClpB* had no impact on the heat-induced expression of chloroplast small HSP and HSP60.

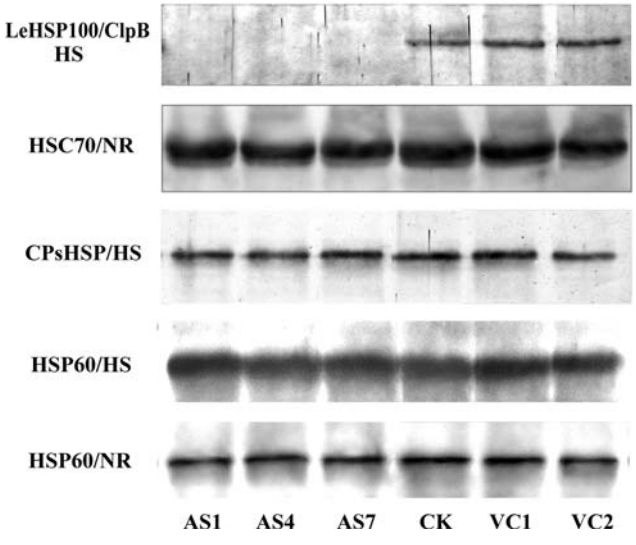

Fig. 6 The protein levels of some HSP in untransformed (CK), antisense transgenic (AS), and pROK II-transformed control (VC) plants. The soluble proteins were extracted from plants, grown at 28°C (NR) or treated at 38°C for 2 h (HS). The immunodetections were preformed using the antibodies against LeHSP100/ClpB, against tomato chloroplast HSP60 β subunit, against tomato chloroplast small HSP and against maize cytosolic HSC70

Indispensability of LeHSP100/ClpB for acquired thermotolerance

After 1-month growth under optimal conditions, all plants grew equally (Fig. 7A), indicating that the repression of heat-induced *Lehsp100/ClpB* and the integration of pROK II vector had no adverse effects on plant growth.

When suddenly exposed to 46°C, all investigated plants displayed the heat-injured symptoms, which were synchronously developed in the course of heat stress. When 2 h of severe heat stress at 46°C were implemented, all investigated plants withered instantly (data not shown). These findings suggested that the repression of heat-induced expression of *Lehsp100/ClpB* did not alter the basal thermotolerance.

When the plants were preconditioned by a series of heat stresses, which were gradually exposed from 38 to 44°C in steps of every 2°C and went on for 2 h at every temperature stage, all stressed plants withstood the lethally high temperature at 46°C for 2 h. On the first day of recovery period, all plants did not show any noticeable difference in phenotypes (7A). Thereafter, the antisense plants displayed gradually the heat-injured symptoms more evidently (Fig. 7B). On the 21st day after heat stress, all of the three antisense lines

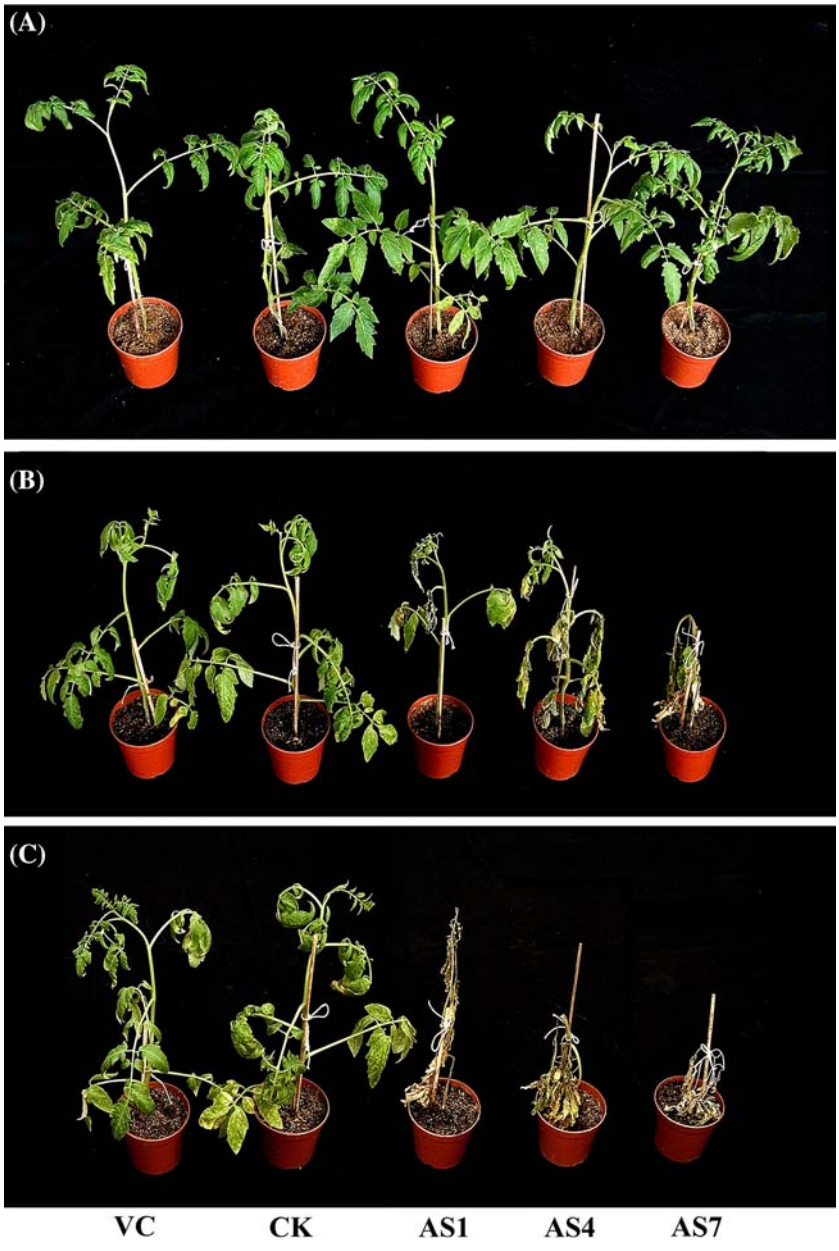

Fig. 7 Phenotypes of thermotolerance in tomato plants. The 6-week-old untransformed (CK), antisense transgenic (AS) and pROK II-transformed control (VC) plants were pretreated at 38°C for 2 h, and were immediately subjected to a more severe heat shock at 46°C for 2 h, and then recovered at 28°C for 1 day (**A**), 14 days (**B**), and 21 days (**C**)

withered, but the control plants remained alive (Fig. 7C). The control plants showed better thermotolerance than antisense lines. These results led to the conclusion that the improvement of thermotolerance by heat acclimation was significant for wild type plants except for antisense lines. In other words, the loss of thermotolerance in antisense lines was closely correlated with the significant repression of *Lehsp100/ClpB* expression during heat acclimation.

LeHSP100/ClpB protected PSII against heat stress

The evaluation of PS II thermotolerance in the investigated plants supported the conclusion drawn on the basis of phenotypes. Before implementing heat stress, the quantum yields (F_v/F_m) in antisense lines and control plants were almost identical, ranging from 0.85 to 0.87. The similarity of quantum yields in PS II suggested undisturbedness of photosynthesis under normal growth conditions by the repression of *Lehsp100/ClpB* expression.

As plants investigated were progressively exposed to higher temperature from 38 to 46°C, in 2°C increase every 2 h, a decrease of F_v/F_m ratios and an increase of F_o values were observed in all investigated plants. Compared with the pROK II-transformed control and wild type plants, antisense lines were markedly impaired on their photosynthetic function, indicated by

both a significant decrease of F_v/F_m values and a drastic increase of F_o values. When the temperature rose up to 46°C, heat stress caused 47.9, 49.3, and 55% increases of F_o values in three antisense lines, respectively, while only 26.8% on average in control plants. Meanwhile, F_v/F_m values in three antisense lines decreased by 18.5, 22.5, and 22.5% while only 11.8% on average in the wild type and pROK II-transformed control lines. Specifically, decreases of F_v/F_m values in antisense lines were in greater extent due to an increase in F_o values, while those in control tomato plants were mainly caused by a decrease in F_m (Fig. 8). These results indicated that the antisense transgenic tomatoes were more vulnerable to high temperature than the control plants.

When heat-stressed plants were returned to 28°C, both antisense and control plants were partially recovered from heat injury. The recovery course had two phases. The rapid rebound of F_v/F_m value occurred in the first 12 h; thereafter only partial reactivation of photochemical efficiency in PSII occurred. The restoration ratios of PS function were significantly different between transgenic and control plants. After 24 h of heat stress, the wild type and pROK II-transformed control plants got 95% recovery in F_v/F_m on average, while F_v/F_m in three transformants have 87.9, 85.5, and 90% of recovery, respectively. During the recovery phase, the F_o value in control plants declined quickly to the normal value, whereas those in the transgenic plants came partially down and remained at a high level even after 24 h. The great severity of irreversible heat-damage to PS II in transgenic plants manifested the important role of LeHSP100/ClpB.

Discussion

In addition to the reported chloroplast HSP100/ClpB in lima bean, the chloroplast HSP100/ClpB-like genes in higher plants can be found by a search in gene database. These putative chloroplast HSP100s are characteristic of predicted transit sequences. This result implies the ubiquity of chloroplast HSP100/ClpB in higher plants, but the corresponding study has rarely been documented. We have cloned and sequenced a tomato gene coding for a ClpB named *Lehsp100/ClpB*. LeHSP100/ClpB protein possesses all the characteristics of the ClpB subfamily, such as the two conserved ATP-binding domains and a spacer region of approximately 300 amino acids between the two NBD domains. Based on the primary structural characters of LeHSP100/ClpB, we defined it as a chloroplast form of HSP100/ClpB. Our experimental data indicated undoubtedly its chloroplast-localization. To our knowledge, this experiment was the first to provide a piece of direct evidence for chloroplast-localization of the HSP100/ClpB subfamily.

Lima bean cp HSP100/ClpB transcript appeared to have a higher constitutive level and demonstrated a weak response to increasing temperature. Unlike lima bean cp HSP100/ClpB (Keeler et al. 2000), neither the transcript nor the protein of *Lehsp100/ClpB* was detectable under optimal growth conditions. Its synthesis was greatly inducible by heat shock. The high response of *Lehsp100/ClpB* to high temperature suggested its close correlation to heat acclimation of plants. The differences in expression profiles of chloroplast HSP100/ClpBs suggest the presence of different regulatory mechanisms in the two plant species.

Photosynthesis is highly susceptible to heat stress. The vulnerability of photosynthesis to elevated temperature is primarily attributed to PSII, since the thermal stability of the other components in chloroplast is higher (Berry and Björkman 1980). Therefore, both the thermotolerance and the repair of damaged PSII reaction centers are the most important ways by which plant cells keep photosynthesis functioning during and after heat stress. In relation to the photochemical efficiency of PS II, F_v/F_m and F_o values have been generally referred to as reliable and quantitative indicators of environmental stresses.

An increase of F_o values can be interpreted as a reduction of the rate constant of energy trapping by PSII centers (Havaux 1993), which could be the result of a physical dissociation of light-harvesting complex from PSII core as it has been observed in several plant species as a result of heat-damage (e.g., Armond et al. 1980). Furthermore, a close correlation has been found between change in F_o and necrosis test after heat stress in a large number of plant species (Bilger et al. 1984). In many plant species, F_v/F_m decreases and F_o increases with rising temperatures (Yamane et al. 1997). In view of the chloroplast-localization of LeHSP100/ClpB, we focused the research on the thermotolerance in PS II. Our experimental data indicated that PS II in antisense lines suffered severely from irreversible-heat damage. Throughout the heat stress, the antisense lines were apparently injured so badly that they lost the recoverability completely and finally withered after 3 weeks. In contrast, the control plants were still alive, temporarily. Similar results were observed from the study on a ClpB mutant of *Synechococcus* sp. In the ΔClpB strain, the loss of ClpB synthesis caused a great decrease in the capacity of the heat acclimation of PS II and even a fivefold reduction in cell survival after a severe heat shock (Eriksson and Clarke 1996).

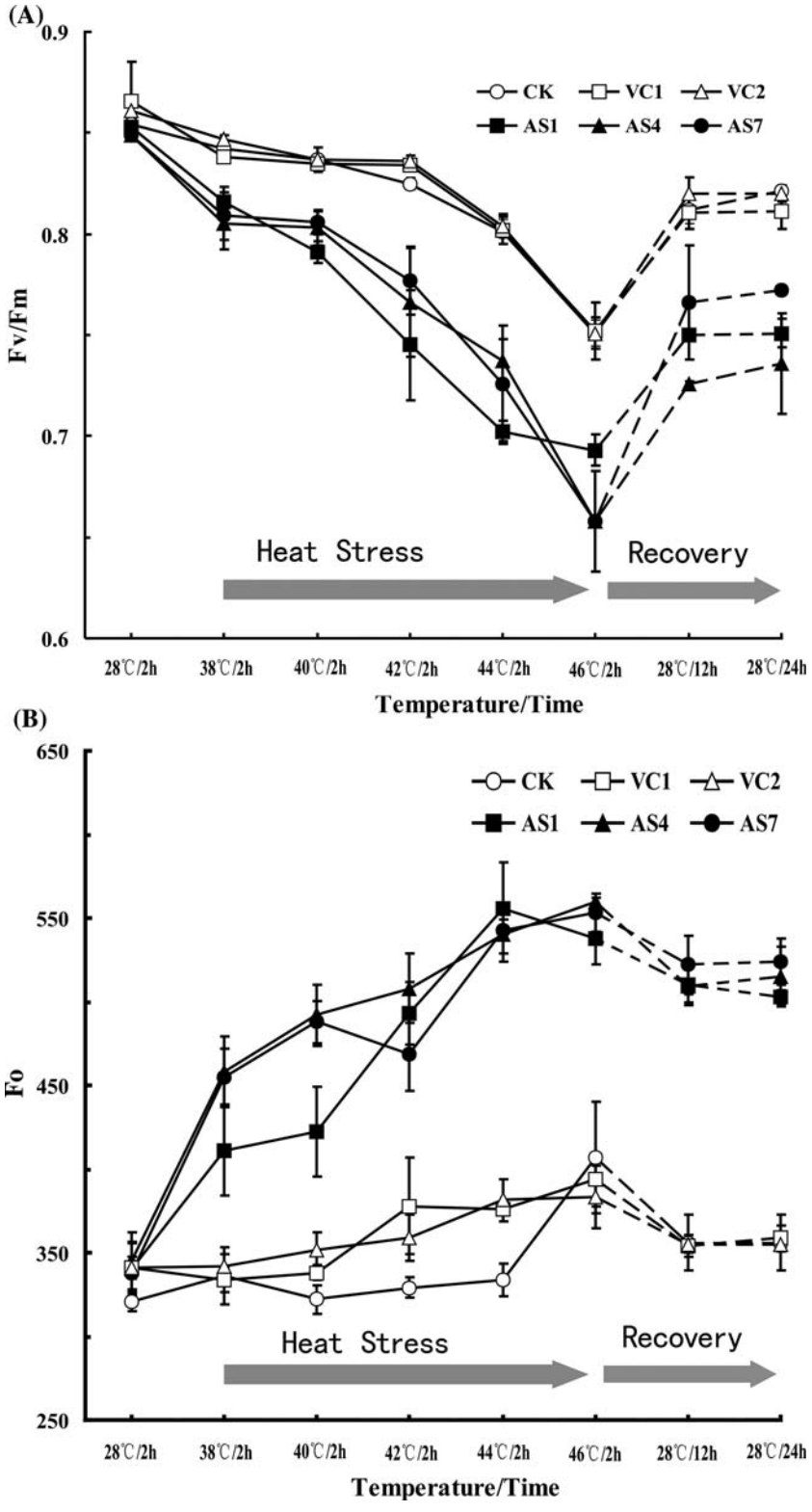

Fig. 8 Effect of high temperature on F_v/F_m and F_o in tomato leaves. The plants, including antisense transgenic plants (AS), pROK II-transformed control (VC) and untransformed plants (CK), were continually exposed to the increasing temperatures, from 38 to 44°C and in steps of every 2°C. At each temperature stage, heat stress was retained for 2 h and then F_v/F_m and F_o were measured (the real lines). After the heat treatment described above, the plants were shifted to normal growth conditions (28°C) for recovery and F_v/F_m and F_o were measured (the dashed lines). The left and right arrows indicates the intervals of heat stress and the recovery

Cyanobacteria are photoautotrophic prokaryotes that are phylogenetically and physiologically related to chloroplasts of photosynthetic eukaryotes (Eriksson and Clarke 1996). The similarity of physiological roles between LeHSP100/ClpB and *Synechococcus* ClpB suggested a functional similarity.

The cytosolic/nuclear ClpB involvements in the acquisition of thermotolerance have been demon-

strated in yeast (Sanchez and Lindquist 1990; Kawai et al. 1999; Nieto-Sotelo et al. 2002), cyanobacterium *Synechococcus* (Eriksson and Clarke 1996), and plants (Hong and Vierling 2000; Queitsch et al. 2000). The physiological role of LeHSP100/ClpB presented here suggests that the thermotolerance is related not only to cytosolic/nuclear ClpBs but also to those in the chloroplast. Numerous studies from other laboratories have already documented a correlation between induction of various chloroplast HSPs and adaptation to heat stress in plants. The stromal HSP70B protein in *Chlamydomonas reinhardtii* is involved in resistance to photoinhibition (Schroda et al. 1999). In tomato cultivars that differ in heat tolerance, there is a positive correlation between the ability of the photosynthetic system to withstand heat stress and the level of CP-sHSP accumulation (Preczewski et al. 2000). An accumulation of the additional CP-sHSP isoforms in creeping bentgrass is genetically linked to thermotolerance (Wang and Luthe 2003). Recent data indicate that chloroplast small HSP protein protects photosystem II from oxidative stress (Neta-Sharir et al. 2005). In this experiment, we have demonstrated that the inhibition of *Lehsp100/ClpB* expression during conditioning pretreatments has disastrous effects on acquired thermotolerance in PS II and even on the whole plant, suggesting chloroplast HSP100/ClpB is another protein that was manifested to involve in plant thermotolerance. Although LeHSP100/ClpB is involved in the thermotolerance, we do not think it act independently in chloroplast. Direct evidence from *E. coli* manifested a functional triade of sHsp, ClpB, and DnaK in reversing protein aggregation *in vivo* (Mogk et al. 2003). The genetic analysis revealed the cooperation between HSP101 and cytosolic small HSP in *Arabidopsis* (Lee et al. 2005). The possibility of cooperation between LeHSP100/ClpB and other chloroplast HSPs could not be ruled out.

Although the HSP100/ClpB proteins are a family with a great diversity of functions, a common ability to disassemble protein aggregates unifies the molecular functions of this diverse family (Schirmer et al. 1996). *Escherichia coli* ClpB can restore thermotolerance to the *Synechococcus ClpB* inactivation mutant (Eriksson and Clarke 2000) and cytosolic/nuclear ClpB from higher plants can confer thermotolerance to yeast (Lee et al. 1994; Schirmer et al. 1994), suggesting that ClpB homologs share the functional characteristics of molecular chaperones in the resolubilization of protein aggregates that accumulate during severe heat stress. Considering the high similarity in sequences between LeHSP100/ClpB and other HSP100/ClpBs, we can reasonably propose that LeHSP100/ClpB also function to facilitate the reactivation of proteins aggregated by heat stress.

The high temperature impacted various aspects of photosynthesis, such as a redistribution of Rubisco from active to inactive forms (Law and Crafts-Brandner 1999; Salvucci and Crafts-Brandner 2004), and the dissociation of the chlorophyll a/b light-harvesting complex from the PS II complex (Armond et al. 1980). As to the chloroplast HSP100/ClpBs in higher plants, protection mechanism against heat stress and their targets remain unclear. Our future research interest is to determine the activity of the *Lehsp100/ClpB* gene by complementation experiments in yeast, its role in resistance to high light stress and its cooperation with other HSPs in chloroplast.

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