J. Plant Biotechnology(2001) *Vol. 3(1). pp. 7~12*

Journal of Plant Biotechnology

Analysis of Thermotolerance in Hot Pepper Using the Antiserum Against Carrot HSP17

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Key Words: antibody, heat shock, hot pepper, HSP, thermotolerance

Abstract

An antiserum against the carrot HSP17 (17 KDa heat shock protein) was raised using the HSP17 purified after being expressed in a recombinant *E. coli* in order to develop an assay system for thermotolerance in crops. The DCHsp17.7 including the coding sequence corresponding to a carrot HSP17 protein was recombined within pET-32 (b) vector and achieved a maximum expression in 4 hours after an induction in *E. coli*. The purified DCHsp17.7 was used as an antigen to generate the corresponding antibody. The polyclonal antiserum was confirmed for it's specificity only to the low molecular weight (lmw) HSP. Besides, the possibilities to use the antiserum to interact with lmwHSPs from other plants such as rice, cucumber, tomato, and hot pepper were examined to be plausible.

To reveal any specific correlation between the amounts of lmwHSP expressed upon HS conditions and an acquisition of thermotolerance two different approaches have been applied. First, it has been shown that only the pre-HS conditions inducing the synthesis of HSP17 allowed for the seedlings to achieve an thermotolerance and to survive the following lethal condition. Second, a western analysis using 15 different collected lines of hot peppers was performed to distinguish each other in terms of the amount of lmwHSP. The results indicated that all 14 hot pepper lines were able to synthesize HSPs in response to an exposure to HS conditions and the amounts of the proteins synthesized at different HS temperatures were variable among the lines. There are several different patterns of lmwHSP synthesized as a function of temperature increase observed and their correlation to physiological aspects of thermotolerance remains to be analyzed.

Introduction

The heat shock response is a highly conserved phenomenon among all organisms so far studied. In plants the environmental stress like heat shock (HS) is one of the major factors to determine growth and reproduction in nature due to their inherent characteristic of immobility compared to animals (Chen et al., 1982; Vierling, 1991). Since Yarwood (1961) first reported the heat shock responses in pea, cucumber and tobacco, it has been discovered that all the plants examined showed a typical HS response upon an exposure to higher temperatures, about 10-15℃ above the optimal temperature for growth (Hendershot et al., 1992). The responses are repression of nonrelevant genes and induction of small sets of genes, called HS genes. The expression of such heat inducible genes provided an acquired thermotolerance which confer the plants to survive at lethal temperatures by a pre-exposure to sublethal temperatures (Heuss-LaRosa, 1987; Hwang, 1993). It was reported that mature plants, seedlings, explants and even cultured tissues were able to show the same responses to HS (Lindquist, 1986; Hwang and Zimmerman, 1989; Hwang, 1995).

In general, the HSPs are involved in generation of thermotolerance in several different ways like unfoldase

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Received Apr. 15, 2000; accepted Nov. 20, 2000.

for mal-structured proteins, stabilizer for membrane structures or other subcellular structures, etc. Loomis and Wheeler (1982) found that a mutant slime mold (*Dictyostelium discoidenm*) lacking the ability to synthesize the lmwHSP could not establish thermotolerance. Sanchez et al. (1990) also showed that a yeast cell required a HSP synthesis to achieve thermotolerance. Thermotolerant varieties were able to synthesize more HSPs than thermosensitive variety (Fender and O'Connell, 1989). Temperature sensitive mutant carrot cells unable to complete embyrogenesis with an exposure to restrictive temperature showed a reduced amount of lmwHSP synthesized (Schnall et al., 1991). There were the positive correlations of 16, 17, 26 KD lmwHSPs with thermotolerance observed in wheat (Krishnan et al., 1989). In addition, it was shown that constitutive expression of the chloroplast small HSP gene increases thermotolerance in transgenic tobacco (Lee, 2000).

Regardless of developmental stages, all plant tissues examined so far showed the similar responses to HS. In facts, there was no variation throughout developmental stages when Hwang et al. (1982) measured thermotolerance in the leaf of soybean, potato, pea, and tomato. Fender and O'Connell (1989) also showed a similar pattern of HSP synthesis in vegetative stages including seedling stages as well as reproductive stages in cotton plants. Therefore it may be possible to develop a method to estimate thermotolerance of plants by measuring the amount of HSPs in young seedling. For that purpose an immunological method to quantitate the amounts of lmwHSP was developed and examined the possibility of its application.

Materials and Methods

Plant materials

The 14 lines of hot pepper provided by Chung Ang Seed Co. Ltd. are following; 320A (Chang-gae), 334A (Do), 104I (Milyang Native), 1358 (Cheju Native), 118H (Chungson/Dopyong Native), 107D (Goesan Native), 108L (Junggongcho), 171A (Kyeongsan Native), 171M (Sangju Native), 231E (Thailand Native), 275F (Malaysia), 89P31 (Lorai), 89P32 (BG-1), 95P151 (Keriting). They are supposed to be variable in terms of thermotolerance based on field examination. For general characterization of the HSP synthesis, DaeMyoung was used. The seeds were germinated to be 4 cm long in dark condition before treatment. A treatment of heat shock was done with plants in an incubator at 40℃ for 3 hours.

Protein extraction from hot pepper seedlings

After an exposure to the defined heat shock temperatures (28, 40, 44, 48℃) for 3 hours, the seedlings were collected and freezed quickly in liquid nitrogen. Grinding was performed with pre-chilled mortar and pestle. To the powdered tissues the same volume of 2X sample buffer (0.125 M Tris-HCl pH 6.8, 4% SDS, 20% Glycerol, 10% β-Mercaptoethanol, 0.0025% Brilliant blue R-250) was added and homogenized before centrifugation at 16,000 g for 15 minutes at 4℃ and then the supernatant was saved.

Synthesis of carrot HSP17 in *E. coli*

A 750 bp fragment containing a carrot DChsp17.7 (Darwish et al., 1991) was recombined to pET-32 (b) vector (Novagen) at *Bam*HI and *Sal*I sites. The resulting recombinant construction (pHSG) was transformed into BL21 (DE3). A 50 μL of overnight-cultured BL21 cells containing a pHSG was used to innoculate 5 mL of LB and 1 hour and 45 minutes after the innoculation, IPTG was added to induce an expression of the recombinant gene to produce the fusion protein including DChsp17.7.

Extraction of Proteins from *E. coli*

A pellet of the *E. coli* producing DChsp17.7 resulting from centrifugation at 3,800 g for 10 minutes was dissolved in 1/10 volume of buffer (50 mM Tris-HCl pH 8.0, 2 mM EDTA), and 100 μg/mL lysozyme and 1% Triton X-100 were added before incubation at 30℃ for 15 minutes and then homogenized. The homogenate was centrifuged at 16,000 G for 15 minutes at 4℃ and the supernatant was applied to 15% SDS-PAGE to see the DChsp17.7 synthesized. The fused protein of DChsp17.7 with thioredoxin was treated with enterokinase (Novagen) at 21℃ for 16 hours to cleave off thioredoxin from DChsp17.7.

Purification of HSP from the gel

The fusion protein cleaved by enterokinase was separated in 15% SDS-PAGE and the band of DChsp17.7 was cut out and ground to make a fine powder on a glass plate. The powder was homogenized in elution buffer (50 mM Tris-Cl pH 8.0, 0.1% SDS) and then shaken at 4℃ for 12 hours. And then centrifugation to remove gel debris was done at 3,800 g at 4℃ for 10 minutes and 4 times volume of acetone was added to the supernatant for precipitation at −20℃ for 16 hours. The precipitated protein was collected by centrifugation at 16,000 g for 20 minutes at 4℃ and

dried and then dissolved in PBS (phosphate-buffered saline).

Synthesis of polyclonal antiserum against HSP17

Mixtures of 100 μg of purified HSP and 500 μL of Freund's complete adjuvant were used for immunization of rabbit twice at 2 weeks interval and another 100 μg of HSP was injected after 2 weeks for boosting up. Two weeks after the third injection total serum was collected. A centrifugation at 2,760 g for 10 minutes at 20℃ after incubation at room temperature overnight was done to provide the supernatant containing polyclonal antibody against HSP17. A pre-immune serum was also collected before the immunization and used as a control to confirm the specificity of the post-immune antiserum for western analysis.

Quantification of proteins

The proteins extracted from plant tissues were dissolved in PBS (phosphate-buffered saline) and then quantitated by using a Bicinchoninic Acid Protein Assay kit (Sigma) and spectrophotometer (Unicam).

Western analysis

Proteins equivalent to the same fresh weight of tissue were separated in 15% SDS-PAGE. Electroblotting was done using nitrocellulose membrane (Schleicher & Schuell) and electroblotter (BioRad) at 70 V for 2 hours. The blotted membranes were dried at room temperature for 60 minutes and blocked in blocking solution (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk) for 45 minutes with shaking. Blocked membrane was washed twice with TBST (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20) and primary antiserum (1/100 dilution) was added and shaked for 30 minutes. And after another washes with TBST for 20 minutes four times, the alkaline phosphatase (AP)-conjugated secondary antibody (purchased from Promega and diluted to 1/7,500) was added and incubated for 30minutes. The membrane washed with TBST for 10 minutes was air-dried to get gentle dryness and color reaction was started with 10mL AP development solution (100 mM Tris-HCl pH 9.5, 100 mM NaCl 5 mM $MgCl_2$, 50 mg/mL nitroblue tetrazolium, 50 mg/mL 5-bromo-4-chloro-3-indolyl phosphate) added.

Results and Discussion

Production of carrot HSP17 in *E. coli* and purification

To obtain a large amount of plant HSP17, a 750 bp DNA fragment containing the part of coding sequence and 3'-untranslated region of DChsp17.7 was recombined into a pET-32 (b) expression vector (Figure 1). The synthesis of T_7 DNA polymerase was induced by an addition of IPTG, which leads to transcribe the fused genes of DChsp17.7 and thioredoxin from a T_7 promoter. A maximum level of expression for the fusion protein of 34 KDa was observed in 4 hours after an addition of IPTG (Figure 2A). When the enterokinase was used to cleave a site between thioredoxin and HSP17.7 in the fusion protein it appears that an 16 hours' digestion was the most efficient to give rise to a DChsp17.7 of 15.25 KDa and a thioredoxin of 17.23 KDa (Figure 2B). The HSP17 separated in SDS-PAGE were eluted from the gel and purity of the eluted protein was confirmed from SDS-PAGE analysis. A single band appeared from silver staining of the gel may indicate a high efficiency in purification of the HSP17 (data not shown).

Western analysis showing a specificity of the HSP17 antiserum

The specificity of the antiserum raised against HSP17

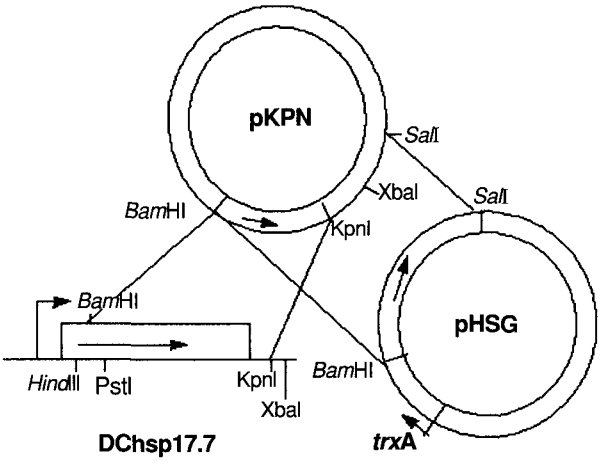

Figure 1. Schematic diagram showing the construction of pHSG from a ligation of a *Sal*I and *Bam*HI fragment of pKPN into pET-32 (b), an expression vector. The pKPN contains parts of coding sequence and 3'-UTR of DChsp17.7 gene. DChsp17.7 is a member of the genes encoding for a low molecular weight heat shock protein (17.7 KDa) in carrot. The *trx*A represents for a gene encoding thioredoxin (17.23 KDa) from *E. coli*.

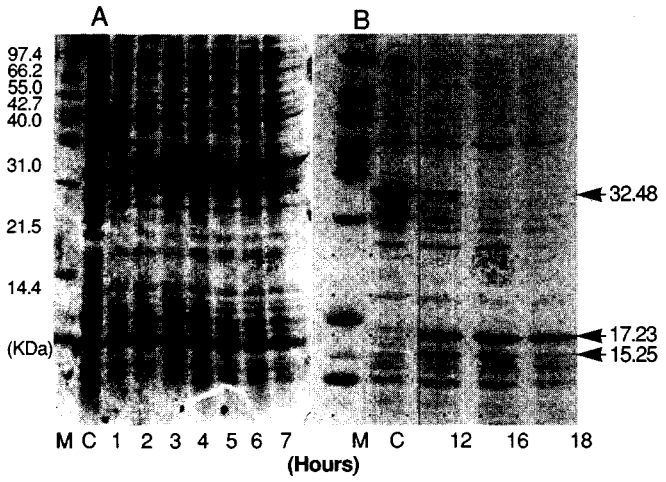

Figure 2. (A) Synthesis of carrot HSP17 in *E. coli* as an incubation time increases up to 7 hours after induction with IPTG. A new band of 32.48 KDa appeared upon an addition of IPTG represents for the fusion protein of both carrot HSP17 and thioredoxin. M: marker; C: no IPTG; 1-7: hours after IPTG treatment. (B) The total proteins including plant HSP17, from pHSG cells, digested with enterokinase that is expected to cleave off the HSP17 from thioredoxin. M: marker; C: total proteins of pHSG cell without digestion with enterokinase; 12, 16, 24: the same protein as above with digestion for different incubation time (hours).

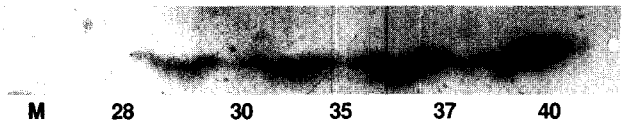

Figure 3. Western analysis showing the HSP17 accumulated as increase the temperature. M: lane for molecular weight markers; 28, 30, 35, 37, 40: temperatures (℃).

was shown by western analysis (Figure 3) using the proteins extracted from the hot pepper (Daemyoung) seedlings incubated at 28, 30, 35, 37, and 40℃. The western analysis showed a specific interaction between a group of HSP17 and the antiserum raised against carrot HSP17. In addition the serum obtained before immunization showed no signal that may indicate the specificity of the antiserum (data not shown). The western analysis shown in figure 3 indicated that the HSP17 was detected as low as at 30℃ and the amount of the HSP17 was increased gradually with a peak at 40℃. It appears that the seedling of Daemyoung shows an optimal temperature for a maximum synthesis of HSP17 at 40℃. However it may not be possible to use these parameters to generalize the heat shock response for various lines of hot pepper since there were many patterns of the induction observed as a function of HS temperatures among different varieties of hot pepper (will be shown in Figure 7).

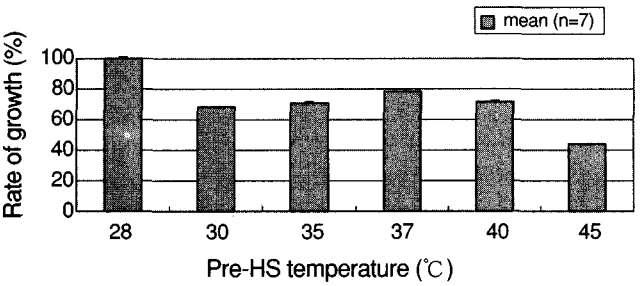

Figure 4. Acquired thermotolerance by pre-HS at various temperatures as shown by relative growth of hot pepper seedlings; Seedlings were treated at various temperatures for 2 hours and recovered at room temperature for 1.5 hours before exposure to 45℃ for 0.5 hour. The length of seedlings increased were measured 1 week after the treatment and the relative growth were obtained by dividing the growth of the seedlings having a pre-exposure at an indicated temperature with a 45℃ treatment by the growth without a 45℃ treatment.

Stability of HSP17 after synthesis and involvement of HSP17 in acquisition of thermotolerance

In order to see a role of HSP17 in development of thermotolerance, hot pepper seedlings were pre-heat shocked at 30, 35, 37, 40℃ for 2 hours and then allowed to recover at room temperature for 1.5 hours. And then the seedlings were exposed to 45℃ for 0.5 hour which is shown to be a lethal condition, to see what degree of the pre-heat shock condition was enough to develop thermotolerance for survival. Compared to the seedling grown at 28℃ without any treatment, the seedlings with a pre-exposure to 37℃ showed a higher level of growth than seedlings pre-heat shocked at 30, 35, and 40℃ (Figure 4). This may indicate that a certain level of HSP17 accumulated as shown in figure 3 is necessary for the seedling to establish a certain level of thermotolerance to survive the following lethal condition. It has been observed that the HSP17 once synthesized was stable for at least 4 days (Figure 5). In addition to the correlation observed between the stability of HSP17 and state of thermotolerance, many other evidences suggesting essential roles of HSP17 in thermotolerance in plants (Lin et al., 1984; Hwang, 1993; Malik et al., 1999) could lead to a conclusion that the HSP17 is responsible for an acquisition of thermotolerance in plants. Based on this conclusion it may be resonable to assume that an ability to synthesize the HSP17 is correlated to level of thermotolerance acquisited in plants.

Cross reactivity of carrot antibody to HSP17s from various plant species

The plant HSP17 belongs to a multigene family in

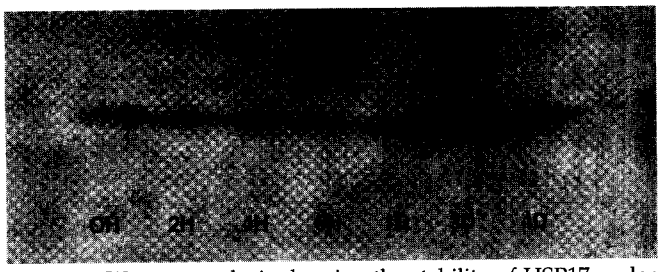

Figure 5. Western analysis showing the stability of HSP17 under non-HS condition once synthesized upon HS. H: hours; D: days.

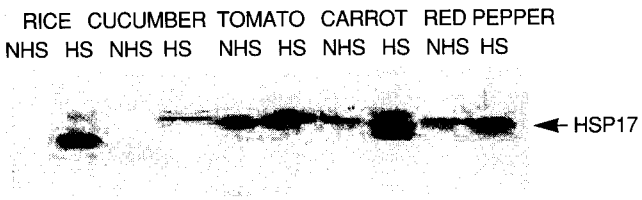

Figure 6. Western analysis showing the specific cross-reactions of the antiserum raised against the carrot HSP17 synthesized in *E. coli* to the HSPs from various plants.

which members are so homologous and conserved in both nucleotide and amino acid sequences in various plants (Key et al., 1985). Therefore the proteins extracted from 5 different plant species after a heat shock treatment were examined to check any possible cross-reaction with the antiserum raised against carrot antigen. All plant species tested, including rice, cucumber, tomato, carrot and hot pepper were shown to react with the antiserum as shown in Figure 6. The basal levels of HSP17 under non-HS conditions were also observed in tomato, carrot, and hot pepper. In case of the carrot there were 3 bands shown to react to the antiserum. This is not unusual since carrot had been shown to produce 10 HSPs in 2D gel analysis to cross-react to the antiserum raised against to the tomato HSP17 antiserum (Hwang, 1993). This result may indicate the antiserum raised against carrot HSP17 can be used for various plant species including hot pepper to quantitate the amounts of HSP17 synthesized under various conditions of either HS or other stresses.

Variation in the pattern of HSP17 synthesis among 14 lines of hot pepper as temperatures increases

The 14 different lines of hot pepper were analyzed in an attempt to see any difference in pattern of expression for HSP17. Based on the western analysis shown in Figure 7-A, it may be possible to classify them into 5 different types based on the pattern of HSP17 expression at 28, 40, 45, and 48℃ as summarized in Figure 7-B. The first group

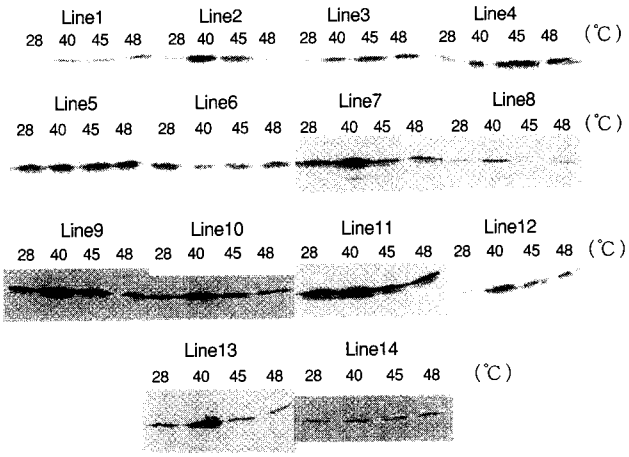

Figure 7-A. Comparative western analysis of the lmwHSPs extracted from the seedlings of 14 different lines of hot pepper after an exposure at 4 different temperatures. Line 1 to 14 represent for hot pepper varieties of 320A (Chang-gae), 334A (Do), 104I (Milyang Native), 1358 (Cheju Native), 118H (Chungson/Dopyong Native), 107D (Goesan Native), 108L (Junggongcho), 171A(Kyeongsan Native), 171M (Sangju Native), 231E (Thailand Native), 275F (Malaysia), 89P31 (Lorai), 89P32 (BG-1), and 95P151 (Keriting), respectively.

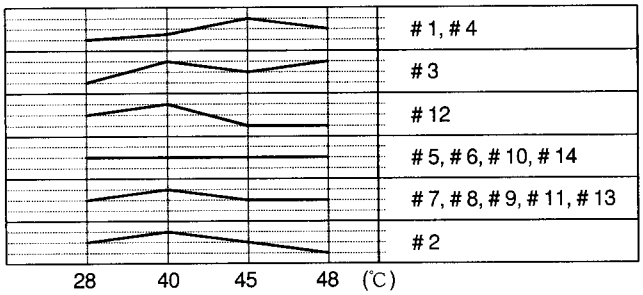

Figure 7-B. Grouping of 14 hot pepper lines based on the relative amounts of lmwHSP synthesized at different temperatures.

including the lines of 1 and 4 showed a gradual increase as the temperature getting higher. The second group consisting lines of 5 and 6 showed a basal induction at 28℃ and a flat level of expression throughout the higher temperatures. The third group of 2, 10, 11, 14 and 15 showed a broad peak in both 40 and 45℃ and low expression at both 28 and 48℃. The fourth group including lines of 8, 9 and 13 showed a peak at 40℃ and a gradual decrease as the temperature increased. The last group including lines of 7, 12 showed a decreasing level of expression as temperature increased.

Besides, based on the amounts of HSP17 synthesized, it may be possible to rank each lines of hot pepper such that 12, 10, 6, 8, 5, 11, 7, 13, 2, 4, 14, 15, 1, and 9. The above groupings may reflect the inherent properties of ther-

motolerance but the exact correlation remains to be solved until systematic examinations of the physiological aspects of thermotolerance in those lines in field will be performed. In an attempt to correlate the levels or induction patterns of HSP17 observed to the extents of thermotolerance among 14 lines of hot pepper, a careful examination of the various characteristics of the hot pepper that are related to a high temperature stress in the field should be performed. There are many reports showing a close correlation between the low molecular weight HSP and thermotolerance in plants but their comparisons were only based on any relevant difference in the amount of HSP synthesized at a fixed temperature supposed to induce HS response. The data shown in this study indicate that there can be variable patterns in induction of HSP17 as a function of increasing temperature among different lines even within a single species. Further field studies on the physiological characteristics of the plants in response to a high temperature stress will reveal more accurate relationship between degrees of thermotolerance and amounts of HSP17. In addition, it appears that the antiserum raised against HSP17 will provides an efficient tool to estimate thermotolerance of plants in general.

Acknowledgements

This work was supported by ARPC through a research fund on "Breeding of Hot Pepper Varieties for the International Market".

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