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Acquisition of Thermotolerance in the Transgenic Plants with BcHSP17.6 cDNA

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BcHSP17.6 cDNA의 도입에 의한 형질전환된 식물의 내열성 획득

김기용 · 정민섭 · 조진기

摘 要

대만산 배추 (*Brassica campestris* M.)로부터 분리한 BcHSP17.6 cDNA(내열성 유전자)를 pBKS1-1 vector에 subcloning하므로서, NPTⅡ 유전자와 P35S-HSP17.6 cDNA를 가지는 pBKH4 재조합 플라스미드를 제작하였다. 이들 플라스미드를 갖는 *A. tumefaciens* LBA4404로서 담배잎 단편을 24시간 동안 공배양하므로서 감염시켰으며, 이들 유전자로 형질전환된 shoot는 100 μg/ml의 가나마이신을 첨가한 MS-n/B 배지에서 선발하였다. 담배 (*Nicotiana. tabacum*)의 열에 대한 치사온도는 50℃에서 15분 이상이었으며, BcHSP17.6 cDNA를 갖는 형질전환된 식물체는 이 온도에서 내열성을 나타내었다. 식물체의 형질전환 여부는 α-^{32}P로 표지한 BcHSP17.6 cDNA 단편을 probe로 이용해서 Southern blot hybridization을 실시하므로서 확인하였다. BcHSP17.6 cDNA의 발현정도는 Northern blot 분석과 이중면역확산 방법으로 확인하였다. 본 연구에서, 담배에 도입한 BcHSP17.6 cDNA는 내열성과 관련이 있는 유전자로서, HSP17.6 단백질은 식물체를 열에 의한 손상으로부터 방지해 주는 protector 역할을 하는 것으로 사료된다.

Ⅰ. INTRODUCTION

In response to high-temperature stress, plants and other organisms synthesize a discrete set of proteins known as Heat Shock Proteins (HSPs) that are hypothesized to prevent and/or repair stress-induced damage (Lindquist and Craig, 1988). In higher plants, Low Molecular Weight (LMW) HSPs with molecular masses between 15- and 30-kD are the major proteins synthesized during heat stress and can accumulate to more than 1% of total leaf cell protein (Mansfield and Key, 1987; DeRocher et al., 1991; Hsieh et al., 1992). Four gene families of LMW HSPs have been characterized in plants (Vierling, 1991). The class Ⅰ and class Ⅱ families encode cytoplasmic proteins, and the other two families encode endomembrane- and chloroplast-localized proteins. Although there is good evidence for HSPs in the HSP90, HSP70 and HSP60 classes as "molecular chaperones" (Gething and Sambrook, 1992), the function of the LMW HSPs is unknown. It is known, however, that they are critical for plant survival by considering the facts of their evolutionary conservation and presence in three different cell compartments.

In this experiment, we transformed tobacco plants as a model plant with *BcHSP17.6* (Thermotolerance gene, GenBank Accession Number; AF022217), a cytoplasmic class Ⅰ LMW HSP gene derived from Chinese cabbage (*Brassica campestris* M.) by binary vector system of Agrobacte-rium tumefaciens. We confirmed the expression of the introduced gene and the survival of the transformed plants at otherwise lethal temperature.

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Ⅱ. MATERIALS AND METHODS

1. Plant, Bacterial Strains and Plasmids

Nicotiana tabacum cv. Samsun was cultivated in a greenhouse under a regime of 16 hours light (28℃) and 8 hours dark (20℃). The plants were used as a model plant in the transformation experiments.

Bacterial strains used as host organisms for DNA transformation or transfection were *Escherichia coli* HB101, DH5α, and BL21(DE3), and *Agrobacterium tumefaciens* LBA4404.

Plasmid pBLH4, constructed by subcloning BcHSP17.6 cDNA fragment (GenBank Accession Number; AF022217) into the *Bam*H I site of pBluescript Ⅱ SK(+) vector, was used as the source of *BcHSP*17.6 which was the cloned gene encoding class I LMW HSP from Chinese cabbage (*Brassica campestris* M.) cDNA library. The plasmid pBKH4 was constructed by subcloning BcHSP17.6 cDNA fragment from pBLH4 into the *Bam*H I site of pBKS1-1. The binary vector pBKS1-1 is a pBI121 derivative that contains a NPT Ⅱ gene, a selective marker for plant transformation conferring kanamycin (Km) resistance (neomycin phosphotransferase Ⅱ gene braked by the promoter and terminator of the nopaline synthase gene, nos) and a 35S promoter for constitutive expression of transferred insert DNAs in plants. The expression vector, pETH4 was also constructed by ligation of *Nco* I digested pET-3d vector and BcHSP17.6 cDNA. Preparation of competent cells and transformation of *E. coli* were conformed as Dower et al. (1988) and Taketo (1988) methods. The plasmid DNA was transfered into *A. tumefaciens* as described by Holster et al. (1978: freeze thaw method).

2. Media and Culture Conditions

E. coli strains of HB101, DH5α, and BL21(DE3) were grown in Luria-Bertani (LB) medium at 37℃. *A. tumefaciens* LBA4404 was cultured in YEP medium (1% of Bacto-peptone, 1% of Bacto-yeast extract and 0.5% of Sodium chloride) at 28℃. Selective antibiotics used were ampicillin (50 μg/ml), kanamycin (50 μg/ml or 100 μg/ml) and rifampicin (100 μg/ml). Transformed plant tissues were cultivated on Murashige-Skoog medium (MS) supplemented with kanamycin at 100 μg/ml and cefotaxime at 200 μg/ml. For overexpression of 17.6-kD HSP in BL21(DE3), IPTG was added.

3. Transformation of Plant Tissues

Transformation of plant tissues was confirmed as described by Kim et al. (1993). Sterilized tobacco (*N. tabacum* cv. Samsun) leaves were sliced into 1 cm^2 sections and infected with *Agrobacterium* cells harboring pBKH4. After co-cultivation for 24 hours, *Agrobacterium* cells were washed with sterilized MS medium and placed on shoot induction medium containing kanamycin (100 μg/ml) and cefotaxime (200 μg/ml). The transformed tobacco shoots were selected with kanamycin resistance and transferred to root induction medium containing kanamycin(100 μg/ml). Finally, the transgenic plants were transplanted to soil medium.

4. Southern Blot and Nothern Blot Analysis

Genomic DNA was isolated from leaves as described by Murray and Thompson (1980). Genomic DNA was digested with restriction endonucleases and subjected to electrophoresis in 0.8% agarose gels. Total RNA was isolated as described previously (Vierling et al., 1986), then subjected to electrophoresis in 1.2% agarose gels. DNA and RNA were blotted on to Hybond-N+ nylon membranes (Amersham Co) in accordance with standard protocols and hybridized with labeled HSP17.6 cDNA, respectively. ^{32}P labeled HSP17.6 cDNA probes were prepared as described by Krug and Berger (1987). Hybridization was performed at 42℃ overnight in 50% formamide, 10% dextran sulfate, 1% SDS, 0.1 ㎎/㎖ denatured salmon testis DNA, and labeled probes (5x106 cpm/㎖). Then the filters were washed twice in 2× SSC-0.1% SDS for 30

minutes and once in 0.1×SSC-0.1 % SDS for 30 minutes at 42℃.

5. Antibody Preparation

Antibody against cabbage HSP17.6 was generated using antigen expressed protein in *E. coli*. For preparation of antigen, 17.6-kD HSP band identified by Coomassie blue staining was cut out of the gel separately and washed thoroughly with distilled water. The isolated protein was used to generate antibody in New Zealand white male rabbits. Two weeks after the final injection, 20 ㎖ of blood was drawn from the ear vein and allowed to clot at room temperature for 1 hand then left overnight at 4℃. The serum was collected by centrifugation and used as polyclonal antibody.

Prepared antibody was confirmed by double immunodiffusion method (Ouchterlony and Nilsson, 1986).

6. Protein Isolation and Ouchterlony Double Diffusion

Total protein extracts were prepared following the method of Hurkman and Tanaka (1986) with slight modifications. Tissues were ground to a fine powder (500 ㎍) and was adjusted in a microfuge to 600 ㎕ with 0.7M sucrose, 0.5M Tris-HCl, pH8, 5mM EDTA, 0.1M NaCl, 300mM 2-mercaptoethanol, and 2mM PMSF. Phenol was added and the mixture was vigorously shaken at room temperature for several minutes. Two phases were separated by centrifugation and the upper phenol phase was collected. Proteins were precipitated from the phenol phase with 5 volumes of 0.1M ammonium acetate in methanol at -20℃. After centrifugation, the pellet was washed three times with methanolic 0.1M ammonium acetate and once with acetone. The protein was solubilized in 50mM Tris-HCl, pH6.8, 100mM DTT, 2% SDS and 10% glycerol, and stored at -20℃.

Double diffusion was performed as described by Ouchterlony and Nilson (1986). One hundred ㎖ of 1% agarose in the 0.5xTBE buffer was heated slowly to dissolve the agarose. It was distributed into the plastic 90mm diameter Petri dishes and allowed to cool on level bench with lids off. A series of holes in agarose plates was formed by punching with a well-sharpened 3 or 4 mm cork borer. Each well was filled with 50～70 ㎕ of either antibody or protein isolated from transformed tobacco leaves or other samples.

7. Determination of Heat-Killing Temperature

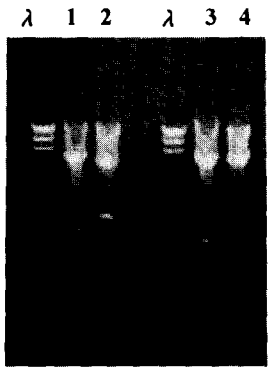
Non-transformed tobacco (*N. tabacum* cv. Samsun) and transformed tobacco with pBKH4 harboring LMW HSP cDNA were grown in a greenhouse. For determination of heat-killing temperature, non-transformed tobacco were planted in flower pots. These plants were grown in a greenhouse for one month, and then were exposed to several steps of high temperatures around 50℃ for 5, 10, 15, 20, 25 and 30 minutes.

Ⅲ. RESULTS AND DISCUSSION

1. Construction of Pasmids pETH4 and pBKH4

To overexpress 17.6-kD HSP, we constructed pETH4 expression plasmid harboring BcHSP17.6 cDNA (GenBank Accession Number; AF022217). This constructed plasmid was confirmed by agarose gel electrophoresis after digestion with *Hind*Ⅲ restriction endonuclease. The plasmids containing BcHSP17.6 cDNA were named pETH4 (sense) and pETH4(－) (antisense) according to the direction of inserts. As expected, 1.16 and o.6 kb bands were observed on *Hind*Ⅲ restriction endonuclease digestion, respectively (Fig 1[A]).

The plasmid pBKH4 was constructed by subcloning *Bam*H I restriction fragment (BcHSP17.6 cDNA) from pBLH4 into the *Bam*H I restriction site of pBKS1-1. The *E. coli* HB101 was transformed with constructed pBKH4 and was grown at the LB medium containing 50 ㎍/ml of kanamycin. *A. tumefaciens* LBA4404 was also transformed with pBKH4. The



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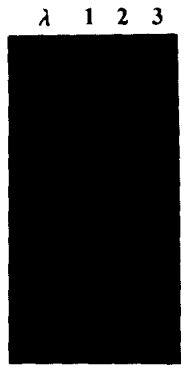


[B]

Fig. 1. Agarose gel electrophoresis of constructed pETH4 and pBKH4 plasmids.
[A] Agarose gel electrophoresis of pETH4 and pETH4(−) plasmids. λ, *Hind*Ⅲ digested λ DNA Marker; Lane 1, *Nco* I digested pETH4; Lane 2, *Hind*Ⅲ digested pETH 4; Lane 3, *Nco* I digested pETH4(−); Lane 4, *Hind*Ⅲ digested pETH4(−).
[B] Agarose gel electrophoresis of plasmid pBKH4. λ, *Hind*Ⅲ digested λ DNA Marker; Lane 1, *Eco*R I digested pBKH4; Lane 2, *Xba* I digested pBKH4; Lane 3, *Bam*H I digested pBKH4.

transformed colonies were selected on YEP medium containing 50 μg/ml of kanamycin and 100 μg/ml of rifampicin. When pBKH4 plasmid was digested with *Bam*H I restriction endonuclease, the BcHSP17.6 cDNA band was observed on 0.8% agarose gel. (Fig 1 [B]). This constructed plasmid pBKH4 was used for production of transgenic tobacco plants with expression of BcHSP17.6 cDNA driven by 35S promoter.

2. Antibody Production and Characterization

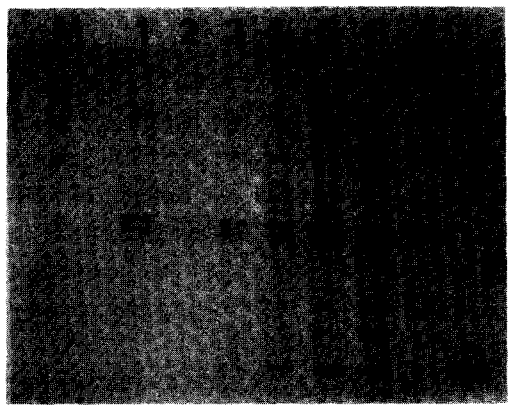
To study the expression of the 17.6-kD HSP in transgenic tobacco plants, antibody specifically recognizing the HSP17.6 protein was generated. 17.6-kD HSP produced in transformed *E. coli* by pETH4 plasmid was used as an antigen.

For overexpression of LMW HSP in transformed *E. coli* with pETH4 plasmid, IPTG was added in LB medium. When overexpressed protein was isolated and ran on the SDS-PAGE gel, 17.6-kD bands were observed at lanes from transformed *E. coli* with pETH4 (from lanes 2 to 6), while it was not observed at lanes from transformed *E. coli* with pET-3d (lane 1) or with pETH4(-) (lane 7) (Fig 2[A]). The arrow indicates 17.6 -kD protein bands. We confirmed that expression of 17. 6-kD protein was observed only at transformed *E. coli* with pETH4.

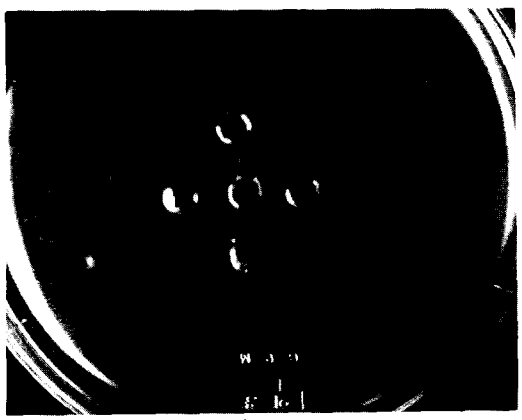
Antibody production was confirmed by double diffusion as described by Ouchterlony and Nilsson (1986), where the precipitin line was observed between antibody and antigen, while not observed at 0.5xTBE buffer including proteins induced from pETH4(-) or pET-3d (Fig 2[B]). The polyclonal antiserum was used in experiments for analysis of the expression of 17.6-kD HSP in transgenic tobacco plants.

3. Transformation of Tobacco Plants

Tobacco leaf discs were cocultivated with the



[A]



[B]

Fig. 2. Expression and antibody production of 17.6-kD HSP.
[A] SDS-PAGE of 17.6-kD HSP in *E. coli* BL21(DE3). M; protein size markers. Lane 1; *E. coli* BL21(DE3)/pET-3d (100 μg of protein). Lane 2-6; *E. coli* BL21(DE3)/pETH4 (50-250 μg of protein). Lane 7; *E. coli* BL21(DE3)/pETH4(-) (100 μg of protein).
[B] Ouchterlony double immunodiffusion patterns of 17.6-kD HSP from the recombinant expression plasmids. The center well (A) contains antiserum against HSP17.6. The surrounding wells contain total protein induced from pETH4 (B), pETH4(-) (C), pET-3d vector (D), and 0.5×TBE buffer (E).

transformed *Agrobacterium* suspension (10^8 cells/ml) for 24 hours and transformed shoots were selected on MS-n/B medium containing 100 μg/ml of kanamycin and 500 μg/ml of carbenicillin. Shoots were induced vigorously from the cocultivated leaf discs but not from the uncocultivated leaf discs. Shoots were also induced vigorously when antibiotics were deleted from MS-n/B medium. Roots were induced better with a half strengh of micronutrients containing 100 μg/ml of kanamycin and 500 μg/ml of carbenicillin. Shoots were induced about 20 days after inoculation whereas it took 35 days for roots (Fig 3). These results were well agreed with the results of Kim et al. (1993). The transformed and regenerated plantlets were transplanted to soil medium for the confirmation of transformation and of the gene expression.

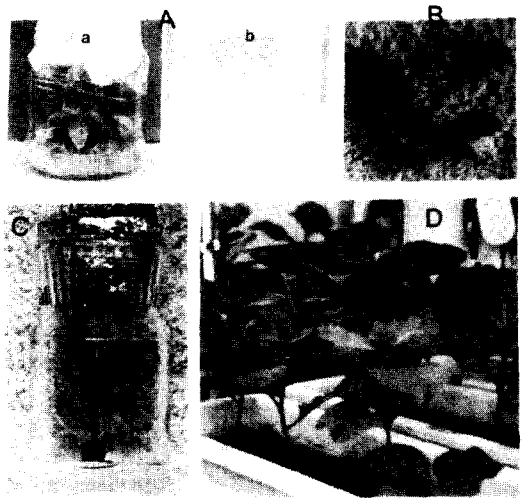


Fig. 3. Regeneration of Transformed Tobacco Plants. A. Shoot emergence; MS-n/B medium containing kanamycin (200 μg/ml) and carbenicillin (500 μg/ml) (a, Cocultivated tobacco; b, Noncocultivated tobacco). B. Root emergence; MS medium with 1/2 volume micronutrients containing kanamycin (200 μg/ml) and carbenicillin (500 μg/ml). C. Regenerated transgenic tobacco plant in glass bottle. D. Transgenic tobacco plants in flowerpot.

4. Confirmation of Tansgenic Tobacco Pants

Transformation of Tobacco plants was confirmed by Southern blot, Northern blot, and Ouchterlony double immunodiffusion. Genomic DNAs were extracted from transgenic and non-transgenic tobacco plant leaves. These genomic DNAs were full-digested with *Bam*H I, and then agarose gel electrophoresed for Southern blot hybridization. When the result was analyzed by Southern blot using α-^{32}P labelled BcHSP17.6 cDNA fragment as a probe, one band was observed at about 0.7 kb range in transgenic plants, while no band was shown in non-transgenic plants (Fig 4 [A]).

We also isolated total RNAs from transgenic and non-transgenic tobacco plants, and Northern blot analysis was performed using the same probe as in Southern blot analysis. In the Northern blot analysis, one RNA band was detected in transgenic plant, but there was no band in control plants (Fig 4 [B]). This suggests that the isolated BcHSP17.6 cDNA was expressed normally in transgenic tobacco plants.

When the expression of BcHSP17.6 cDNA inserted

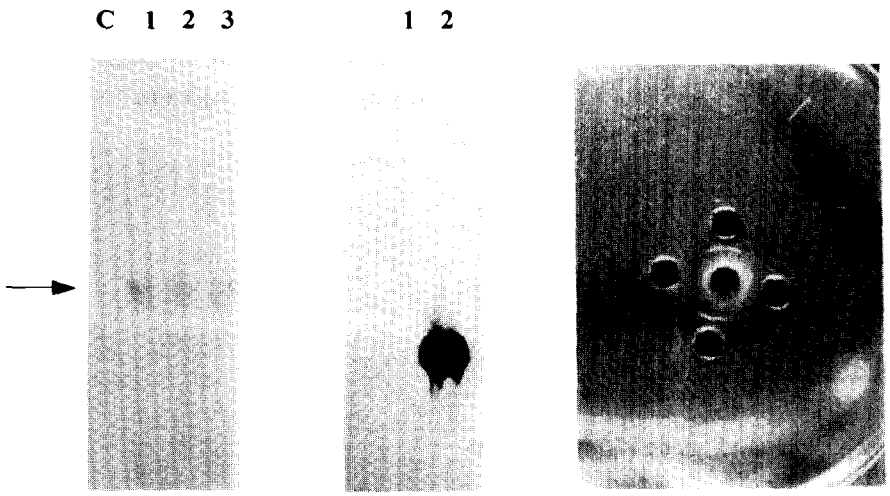


Fig. 4. Southern blot analysis, Northern blot analysis and Ouchterlony double immunodiffusion patterns in transgenic tobacco plants.
[A] Southern blot analysis of genomic DNAs using ^{32}P labeled BcHSP17.6 cDNA fragment as probe DNA. Lane C, nontransgenic tobacco (*Bam*H I digestion; Lane 1-3, transgenic tobacco (*Bam*H I digestion), Arrow indicates BcHSP17.6 cDNA insert bands.
[B] Northern bolt analysis of total RNAs. Total RNAs isolated from transgenic and non-transgenic tobacco plants were electro-phoresed in formaldehyde/1.5% agarose gel, and Northern blot analysis was performed using ^{32}P labeled BcHSP17.6 cDNA fragment as probe DNA. Lane 1, non-transgenic tobacco; Lane 2, transgenic tobacco.
[C] Ouchterlony double immunodiffusion patterns. The center well (A) contains antiserum against 17.6-kD HSP. The surrounding wells contain 0.5 × TBE buffer (B), antigen used for antibody induction (C), total protein extracts of non-transgenic tobacco plants (D), and total protein extracts of transgenic tobacco plants (E).

was analyzed by Ouchterlony double immunodiffusion (Ouchterlony and Nilson, 1986), the precipitin lines were observed both between antibody and total protein isolated from transgenic plants and antigen used for antibody induction, while it was not observed between antibody and other samples containing total proteins isolated from non-transformed tobacco plants (Fig 4 [C]). This suggests that the introduced BcHSP17.6 cDNA was constitutively expressed by 35S promoter in transgenic tobacco plants. This result has already been anticipated by the results of Southern and Northern blot analyses.

5. Determination of Thermotolerance in Transgenic Tobacco Plants

Transgenic and non-transgenic tobacco plants were planted in flower pots and were grown in a greenhouse for 1 month. For determination of heat-killing temperature, only non-transgenic tobacco plants were exposed to 50℃ for 5, 10, 15, 20, 25 and 30 minutes, respectively, and then they were observed for 5 days at 28℃. All the non-transgenic plants treated for more than 15 min at 50℃ died five days later. By this result, we determined the heat-killing temperature of tobacco plants (*N. tabacum* cv. Samsun) to be 50℃ for 15 minutes. According to this result of heat-killing temperature, when transgenic tobacco plants with BcHSP17.6 cDNA were exposed to 50℃ for 15 minutes, they were slightly damaged or not damaged in appearance at all as shown in the Figure 5. Therefore, we suggest that the BcHSP17.6 cDNA introduced to tobacco plants is related to thermotolerance, and that the 17.6-kD HSP acts as a protector from heat damage in plants, that is, the 17.6-kD HSP may act as a 'molecular chaperone''. If this hypothesis is correct, cereal species, vegetables, forages, and the other cultivated plants which are sensitive to high temperature will be better cultivated and produced under high temperature condition by transformation with this BcHSP17.6 cDNA.

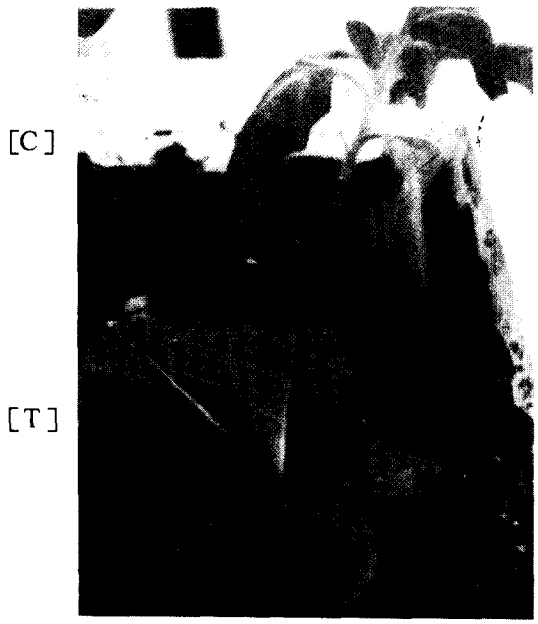


Fig 5. Thermotolerance of tobacco plants transformed with the BcHSP17.6 cDNA. All plants were treated at 50℃ for 15 min. [C] ; Control plant (Non-transgenic). [T] ; Thermotolerant plant (Transgenic).

Ⅳ. SUMMARY

Recombinant plasmid, pBKH4, containing NPTⅡ and P35S-*BcHSP17.6* was constructed by ligation of *Bam* H I -digested pBKS1-1 and *BcHSP17.6* (thermotolerance gene) from pBLH4. The tobacco leaf disc was cocultivated with transformed *Agrobacterium tumefaciens* bearing pBKH4 for 24 hours and transformed shoots were selected on MS-n/B medium containing 100 μg/ml of kanamycin. Heat-killing temperature of *Nicotiana tabacum* was 50℃ for >15min, and transformed tobacco plants with BcHSP17.6 cDNA exhibited thermotolerance at the heat-killing temperature. The transgenic plants were analyzed by Southern blot hybridization with the probe of α-^{32}P labelled BcHSP17.6 cDNA fragments to confirm the stable integration of BcHSP17.6 cDNA. Transcription

and expression level of BcHSP17.6 cDNA were also confirmed by Northern blot analysis and Ouchterlony double immunodiffusion assay. In this study, we suggest that the BcHSP17.6 cDNA introduced to tobacco plant is related to thermoto-lerance and 17.6-kD LMW HSP acts as a protector from heat damage in plants.

V. LITERATURE CITED

1. DeRocher, A.E., K.W. Helm, L.M. Lauzon, and E. Vierling, 1991. Expression of a Conserved Family of Cytoplasmic Low Molecular Weight Heat Shock Proteins during Heat Stress and Recovery. Plant Physiol. 96: 1038-1047
2. Dower, W.J., J.F. Miller, and C.W. Ragsdale, 1988. High efficiency transformation of *E. coli* by high voltage electrophoration. Nucl. Acids Res. 16, 6127-6145
3. Gething, M.-J., J. Sambrook, 1992. Protein folding in the cell. Nature, Lond. 355: 33-45
4. Holsters, M., O.D. Wael, A. Depicker, E. Messens, M.V. Montagu, and J. Schell, 1978. Transfection and transformation of *A. tumefaciens*. Mol. Gel. Genet. 163: 181-187
5. Hsieh, M.H., J.T. Chen, T.L. Jinn, Y.M. Chen, and C.Y. Lin, 1992. A Class of Soybean Low Molecular Weight Heat Shock Proteins. Plant Physiol. 99: 1279-1284
6. Hurkman, W.J., and C.K. Tanaka, 1986. Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol. 81, 802-806
7. Kim, K.Y., D. Son, Y.G. Park, W.I. Jung, and J. Jo, 1993. *Agrobacterium*-Mediated Transformation of a Plant with *Saccharomyces cerevisiae* Acid Phosphatase Gene(PHO5). J. Korean Grassl. Sci. 13: 177-183
8. Krug, M.S., and S.L. Berger, 1987. First-strand cDNA synthesis primed with oligo(dT). Methods Enzymol. 152, 316-325
9. Lindquist, S.L., and E. Craig, 1988. The heat shock proteins. Annu. Rev. Genet. 22: 631-677
10. Mansfield, M.A., and J.L. Key, 1987. Synthesis of the Low Molecular Weight Heat Shock Proteins in Plants. Plant Physiol. 84: 1007-1017
11. Murray, M.G., and W.F. Thompson, 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. 8: 4321-4325
12. Ouchterlony, O., and L.A. Nilsson, 1986. Immunodiffusion and immunoelectrophoresis. In "Handbook of Experimental Immunology(D. M. Weir, L. A. Herzenberg, and C. Blackwell, eds.), Vol. 1, pp. 32.1-32.50. Blackwell, Oxford.
13. Taketo, A. 1988. Sensitivity of *Escherichia coli* to viral nucleic acid. 17. DNA transformation of *Escherichia coli* by electroporation. Biochim. Biophys. Acta. 949, 318-324
14. Vierling, E., M.L. Mishkind, G.W. Schmidt, and J. L. Key, 1986. Specific heat-shock proteins are transported into chloplasts. Proc. Natl. Acad. Sci. U.S.A. 83: 361-365
15. Vierling, E. 1991. The roles of heat shock proteins in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42 : 579-620