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Overexpression of NtHSP70-1 Protects Chlorophyll from High Temperature in Plants

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Heat shock protein 70 (HSP70) is known as molecular chaperone, the fundamental protein participating in various processes, from nascent protein synthesis to protection of proteins during abiotic stresses and developmental programs. However, their biological functions in plants are not yet well known. Here, NtHSP70-1 (AY372069), HSP70 of *Nicotiana tabacum* induced by heat stress was investigated. To analyze the protective role of NtHSP70-1, transgenic tobacco plants, which constitutively overexpressed NtHSP70-1 as well as contained either the vector alone or having NtHSP70-1 in the antisense orientation, were constructed. The altered NtHSP70-1 levels in plants were confirmed by western blotting and transgenic sense lines exhibited tolerance to heat stress. Seedlings with the constitutively expressed NtHSP70-1 grew as green or healthy plants after heat stress. In contrast, transgenic vector or antisense lines exhibited yellowing of leaves or some delay in growth, which finally led to death. Evaluation of chlorophyll contents of heat-shocked transgenic tobacco seedlings indicated that NtHSP70-1 contributes to thermotolerance by preventing chlorophyll synthesis in plants.

Key words : NtHSP70-1, chlorophyll, transgenic tobacco plant, thermotolerance

Introduction

Members of heat shock protein 70 (HSP70) family are known as molecular chaperone, which is the fundamental chaperone in participating in various arrays of processes, from nascent protein synthesis to protection of proteins during abiotic stresses and developmental programs [7,14,18]. HSP70s are found in cellular compartments of almost all organisms. In particular, multiple HSP70s are found in chloroplast of higher plants [12,25]. To date, the best documented function of HSP70 related to chloroplast is based on the finding that cells exposed to heat shock show enhanced resistance to light stress [28]. It suggests that HSP70 helps protection of the photosystem II (PSII) complex from light stress. Excessive light results in irreversible damage to the subunits of PSII reaction centers, which contains a large number of cofactors including chlorophyll [1,29,39,40]. Chlorophylls are essential molecules that are responsible for harvesting solar energy in photosynthetic antenna systems and for charge separation and electron transport within reaction centers [34]. To maintain healthy growth, plants must maintain the entire chlorophyll biosynthesis in good condition, which is executed via a series of cooperative reactions catalyzed by numerous enzymes [3]. In chlorophyll biosynthesis, the synthesis of chlorophyll *a* from glutamate is an important phase, which plays distinct roles during plant development and in response to various stresses [8,9,36,38]. However, the protection mechanism for chlorophyll biosynthesis under stress is not known.

Under stress, maintainenance of photosynthetic activity is determined by the repair of the photodamaged reaction centers and the synthesis of new centers. In particular, the repair of damaged PSII reaction centers is the most important means by which plant cells maintain the function of these centers during and after stress. *In vivo*, HSP70 has been known to participate in the repair and the molecular protection of the PSII reaction centers including chlorophyll biosynthesis [15,16,17,23,26,27]. Repair of PSII induces a series of sequential events leading to its replacement by a de novo-synthesized protein [2,19,21,22]. These results suggest an interaction of the HSP70 with proteins related to chlorophyll biosynthesis. However, there is no direct evidence to indicate that HSP70 protects chlorophyll against stress.

Here, transgenic tobacco plants overexpressing or underexpressing NtHSP70-1 were constructed to investigate a possible role of HSP70 in protection of chlorophyll under heat stress. Overexpression of NtHSP70-1 reduces the degradation of chlorophyll, whereas underexpression causes the opposite effect. Although NtHSP70-1 was not localized in

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chloroplast but nuclear targeting HSP70 (unpublished), this study indicated that NtHSP70-1 confers thermotolerance to plants by indirectly protecting chlorophyll against high temperature.

Materials and Methods

Plant material

Tobacco (*Nicotiana tabacum* L. cv. Wisconsin 38) plants were grown in a growth chamber (26℃, 60% humidity, and 16 hr photoperiod at 200 μE m^{-2} s^{-1} from white fluorescent lamps) or in a greenhouse insulated with a dual door at 26℃ under natural lighting plus some fluorescent lamps to maintain a 16 hr photoperiod. The pBKS1-1 vector, carrying *NtHSP70-1* in the sense or antisense orientation, was transformed to *Agrobacterium tumefaciens* strain LBA4404 by the freeze-thaw method and then used for transforming tobacco according to the procedure of Cho and Hong [6]. Transformants were selected using Kanamycin and then transplanted to soil (T_0 plants). T_0 transgenic plants were grown in a greenhouse insulated with a dual door at 26℃ under natural lighting plus some fluorescent lamps to maintain a 16 hr photoperiod. Those transgenic plants carrying the recombinant construct of *NtHSP70-1* in the sense or antisense orientation, as well as those containing only the vector, were self-fertilized and seeds were harvested (T_1 seeds). T_1 transgenic seeds were allowed to germinate on Murashige and Skoog medium containing 200 μg mL^{-1} kanamycin, and T_1 transgenic lines showing 75% segregation for kanamycin were selected for investigating the function of *NtHSP70-1* in thermotolerance.

Construction of plant expression vector with NtHSP70-1

The open reading frame (ORF) of *NtHSP70-1* was amplified by PCR, with primers encompassing both termini. The 5′ primer was 5′-AAAGGATCCATGGCTCCCGCCGTCGG-3′ and the 3′ primer was 5′-AAAGGATCCTTAGTCGACCTCCTCGACG-3′, with the underlined *Bam*HI restriction sites being introduced. After heating to 94℃ for 5 min, PCR was performed for 33 cycles of 94℃, 1 min; 60℃, 1 min; and 72℃, 2 min 30 s. The amplified PCR product was then digested with *Bam*HI and ligated into the pBKS1-1 plant expression vector [32] at the *Bam*HI site to locate *NtHSP70-1* under the control of the CaMV35S promoter in either sense or antisense orientations. Nucleotide sequencing of the cloned coding region in pBKS1-1 was confirmed by the dideoxy chain termination sequencing method, using a SEQUENASE VERSION 2.0 KIT (Amersham Pharmacia Biotech, USA).

Protein extraction and immunoblotting analysis

For protein blot analysis, 1 g of plant material was ground in liquid nitrogen and resuspended in 1 ml of extraction buffer (50 mM sodium phosphate pH 7.0, 10 mM β-mercaptoethanol, 10 mM EDTA pH 8.0, 0.1% *N*-lauroyl sarcosine, and 0.1% Triton X-100). After 5 min of incubation at 4℃, cell debris was removed by centrifugation for 20 min at 12,000 rpm in a microcentrifuge. A total of 100 μg of soluble protein from each sample was separated on a 12% SDS-polyacrylamide gel for the first dimensional gel electrophoresis. Proteins on the polyacrylamide were electroblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech, USA) in glycine electrode buffer. After preincubation with 5% nonfat milk in TBS (10 mM Tris pH 7.5, 150 mM NaCl), the membrane was incubated with a commercial anti-human HSP70 (HSC70; Stressgen Biotech Corp., USA) diluted 1: 3,000 in 5% nonfat milk in TBS for 1 hr at 25℃ on an orbital shaker [24]. After washing for three periods of 10 min each in TBS containing 0.1% Tween20, the membrane was incubated for 1 hr in goat anti-rabbit IgG conjugated with horseradish peroxidase (Amersham Pharmacia Biotech, USA) diluted 1:5,000 in 5% nonfat milk in TBS. Membrane was washed for three periods of 15 min each in TBS containing 0.1% Tween 20 and developed using an enhanced chemiluminescence kit as recommended by the manufacturer (Amersham Pharmacia Biotech, USA).

Thermotolerance of transgenic seedlings

Experiments to analyze thermotolerance were conducted in controlled environmental cabinets and using seedlings for three distinct transgenic tobacco genotypes: transformed control (pBKS1-1 vector alone), transformed sense (35S-NtHSP70-1 S lines), and antisense (35S-NtHSP70-1 AS lines). Three-week-old tobacco seedlings resistant to kanamycin were transplanted to Petri dishes and moistened with equal amounts of water. Fifty seedlings that were resistant to kanamycin for control, sense, or antisense were exposed to light at 45℃ for 2 hr. After heat treatments, the seedlings were maintained at 26℃, and viability was assessed on a daily basis for as long as 10 days. The results were documented photographically and statistically.

Measurement of chlorophyll accumulation

Seedlings treated with high temperature were placed un-

der continuous fluorescent lighting of 150 μmol m^{-2} s^{-1} at 26℃ and after 0, 1, 2, 3, and 4 days, the content of chlorophyll was measured according to the method described by Oh *et al.* [20]. In brief, 5 mg of seedlings were soaked in 1 ml of 100% ethanol and kept in the dark for 24 hr. Chlorophyll leaked out from the seedlings into ethanol and 500 μl of ethanol extracts were taken from each sample for the measurement of absorbance. Absorbance of the extract was scanned from 400 nm to 750 nm using a spectrophotometer (Thermo Spectronic Co. USA). Chlorophyll content was calculated using a set of equations:

Chl *a* (mg/l) = 13.70 A665 - 5.76 A649

Chl *b* (mg/l) = 25.80 A649 - 7.60 A665

Chl *a+b* (mg/l) = 6.10 A665 + 20.04 A649

Results

Generation of plants with altered HSP70 levels and quantification of HSP70 expression

To identify the function of NtHSP70-1, transgenic tobacco plants with altered levels of HSP70 expression were generated (Fig. 1). The full-length genomic DNA sequence derived from *Nicotiana tabacum* was placed under the control of the constitutive cauliflower mosaic virus 35S promoter (CaM35S) in the sense or antisense orientation. These constructs, or the corresponding vector without an insert, were introduced into plants by selecting the kanamycin resistance marker on the vector (T_0 seedlings). Several independent transgenic lines were selected by RNA blotting, transferred into soil, and grown in greenhouse to generate seeds (T_1 seeds). For selection of individual transgenic lines, seeds were germinated on Murashige and Skoog medium containing 200 μg ml^{-1} kanamycin. In segregation analysis, several selected transgenic T_0 lines appeared to have an integrated T-DNA locus on a single chromosome, since 75% of their T_1 segregating seedlings were resistant to kanamycin. Also, homozygous lines of selected transgenic plants were established by selecting T_1 plants that had exclusively produced kanamycin-resistant T_2 plants after self-crossing.

Selected independent transgenic lines were screened to evaluate HSP70 levels by immunoblotting (Fig. 1). Under normal, nonstressed conditions, levels of the tobacco HSP70 protein in leaves, NtHSP70-1, were significantly higher than those found in the leaves of the pBKS1-1-transformed control and antisense transgenic lines (Fig. 1B and C). Ten transgenic lines for three different transgene constructs were selected

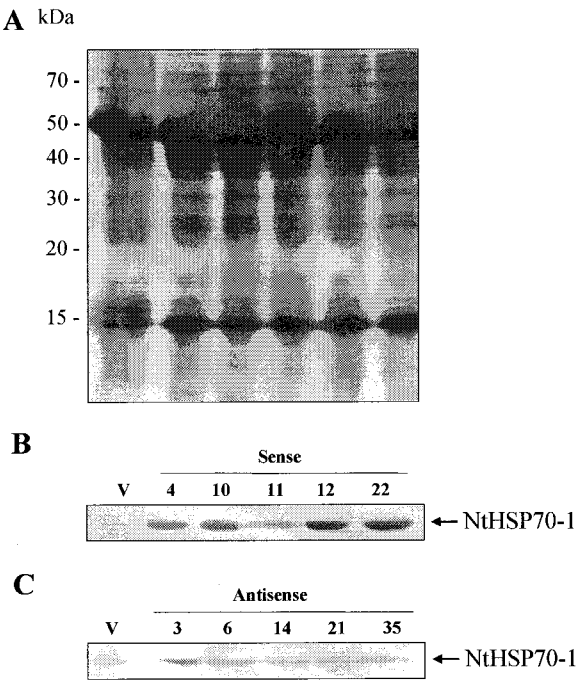

Fig. 1. Confirmation of NtHSP70-1 expression in transgenic tobacco plants. **(A)** Proteins were extracted from transgenic tobacco plants and aliquots of the extracts were analyzed by 12% SDS-PAGE. **(B, C)** Protein blot analysis for NtHSP70-1 produced in transgenic plants under non-heat-stressed conditions. V, transgenic plants carrying only the vector, pBKS1-1; Sense, transgenic plants with NtHSP70-1 in sense orientation; Antisense, transgenic plants with NtHSP70-1 in antisense orientation.

and the selected transgenic tobacco plants did not show any noticeable difference in growth rates compared with non-transgenic tobacco plants.

Thermotolerance can be attributed to NtHSP70-1

Transgenic tobacco plants carrying the recombinant construct of *NtHSP70-1* in sense or antisense orientation and the vector only were self-fertilized, and seeds were harvested (T_1 seeds). T_1 transgenic seeds were segregated on a kanamycin-containing medium, and it was found that 75% of their T_1 transgenic seedlings were resistant to kanamycin, which were used to investigate the function of *NtHSP70-1* in thermotolerance. T_1 seedlings with three different transgene constructs were grown in defined germination medium (GM plates) for 3 weeks and were then subjected to a 45℃ heat shock for 2 hr. The plants were then maintained at 26℃ and their viability was assessed on a daily basis and was photographically recorded (Fig. 2). For each transgene construct, three replicates of fifty seedlings from ten in-

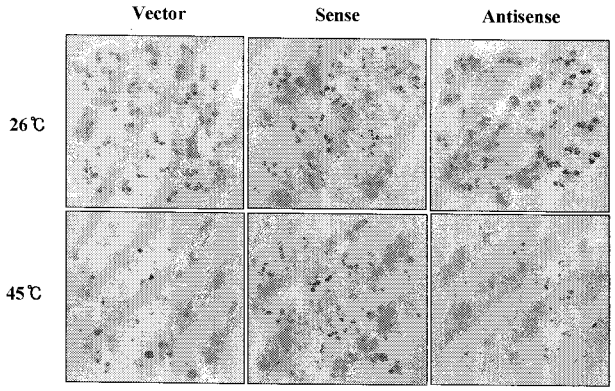

Fig. 2. Thermotolerance of NtHSP70-1 over-expressing transgenic tobacco seedlings. Heat stress (at 45℃ for 2 hr) was induced in kn-resistant 3-week-old seedlings (all at same developmental stage) of pBKS1-1-transformed controls and 35S-NtHSP70-1-transformed tobacco plants in sense or antisense orientation. The seedlings shown were at 10 days since the heat-stress treatments. 26℃, non heat-stress condition. Vector, transgenic plants carrying only the vector; Sense, transgenic plants with NtHSP70-1 in sense orientation; Antisense, transgenic plants with NtHSP70-1 in antisense orientation.

dependent transgenic plant lines were evaluated for their thermotolerance on Day 10 after subjecting the plants to stress. Transgenic seedlings with the constitutively expressed NtHSP70-1 showed almost two times higher survival rate than the transgenic seedlings with the *NtHSP70-1* in antisense orientation and the transgenic seedlings carrying only the vector. However, there was only little statistically meaningful difference in the survival rate between the transgenic tobacco seedlings with the *NtHSP70-1* in antisense orientation and the transgenic tobacco seedlings with the vector only (Fig. 3, Table 1).

Constitutive HSP70 expression prevents chlorophyll breakdown in heat-stressed plants and provides a growth advantage

Effect of NtHSP70-1 in preventing chlorophyll breakdown under heat stress was determined in transgenic tobacco seedlings. Transgenic tobacco seedlings either carrying *NtHS70-1* in sense or antisense orientation and the vector only were exposed to 45℃ for 2 hr and were maintained at 26℃ in light. And then, chlorophyll content was measured on a daily basis for 4 days. Chlorophyll content in the sense transgenic seedlings was approximately 1.6 times and 1.4 times higher than the chlorophyll content in the antisense and the vector transgenic seedlings after 4 days (Fig. 4A).

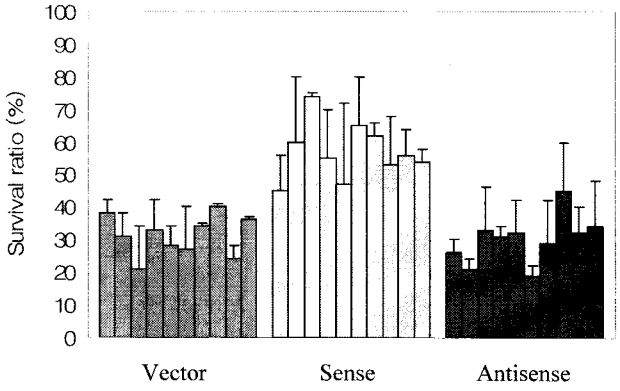

Fig. 3. Elevated levels of NtHSP70-1 conferred thermotolerance to seedlings. Kn-resistant 3-week-old seedlings were treated at 45℃ for 2 hr and then returned to 26℃ and were observed for 10 days. Viabilities were plotted as percentage of number of survived seedlings after heat-treatment relative to number of untreated seedling. Each datum point is average of three replicates, with 50 seedlings per transgenic line and error bar indicate standard error. Vector, transgenic seedlings carrying only the vector; Sense, transgenic seedlings with NtHSP70-1 in sense orientation; Antisense, transgenic seedlings with NtHSP70-1 in antisense orientation. Transgenic lines were as follows; Sense 1, 4, 8, 9, 10, 11, 12, 17, 19 and 22; Vector 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10; Antisense 3, 6, 14, 17, 18, 21, 35, 41, 42 and 43.

Table 1. Effect of over-expressed NtHSP70-1 in tobacco seedlings on their degree of thermotolerance

	Number of survived seedling (% of control)		
	Vector	Sense	Antisense
Non heat-shock	100	100	100
Heat-shock	31.20±2.16*	57.10±3.68*	30.20±2.76*

Heat stress was induced by heat treatment for 2 hr at 45℃ and then seedlings were kept in non heat shock condition (26℃) up to 10 days; afterward, survived seedlings were counted; 99% confidence intervals are shown (N=30)
*$P<0.01$

Measurement of the content of chlorophyll indicated that transgenic tobacco seedlings with the NtHSP70-1 in sense orientation apparently showed significant effect in preventing chlorophyll breakdown compared with the antisense or the vector transgenic seedlings. The position of the absorption peaks of the sense, the vector, and the antisense transgenic seedlings after the heat-stress treatments was basically identical (Fig. 4B, Table 2).

Constitutive NtHSP70-1 expression also provides a growth advantage to heat-stressed plants. After 2 weeks, there was no meaningful difference in the phenotypes of

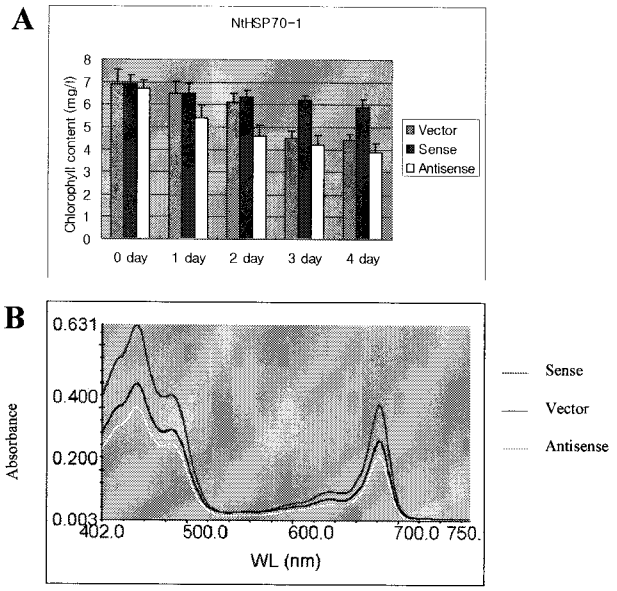

Fig. 4. Comparison of chlorophyll a+b contents in the vector, sense and antisense transgenic seedlings after heat-stress. **(A)** Transgenic seedlings carrying only the vector (Vector), transgenic seedlings with NtHSP70-1 in sense orientation (Sense) and transgenic seedlings with NtHSP70-1 in antisense orientation (Antisense) were heat-stressed for 2 hr at 45℃. Numbers on the X-axis stand for the incubation period under non heat-stress condition (26℃) after the heat-stress treatment. Each datum point is the average of 10 measurements. Error bars represent the standard error. **(B)** Typical absorption spectra of the chlorophyll a from the vector, sense and antisense transgenic seedlings after the heat-stress of 45℃ for 2 hr. After the heat-stress, the seedlings were cultivated under the light for 4 days before the extraction of chlorophyll.

Table 2. Effect of over-expressed NtHSP70-1 in tobacco seedlings on their degree of chlorophyll a+b contents

	Chlorophyll a+b contents (mg/l)		
	Vector	Sense	Antisense
Non heat-shock	6.958±0.718*	6.977±0.536*	6.765±0.652*
Heat-shock	4.403±0.761*	6.383±0.358*	4.297±0.996*

Heat stress was induced by heat treatment for 2 hr at 45℃ and then seedlings were kept in non heat shock condition (26℃) up to 3 days; afterward, chlorophyll a+b contents was determined; 95% confidence intervals are shown (N=10)
*$P<0.05$

plant under nonheat stressed condition. But, most of the heat-stressed seedlings with the antisense gene construct and the vector only exhibited leaf yellowing or some delay in growth and led to death. Whereas seedlings with the constitutively expressed NtHSP70-1 grew as green or healthy plants. On the basis of the level of chlorophyll accumulation, it was found that transgenic tobacco seedlings with the NtHSP70-1 in sense orientation apparently showed significant level of thermotolerance compared with the antisense transgenic seedlings and transgenic seedlings carrying only the vector.

Discussion

In this study, experimental evidences for the role of HSP70 in protecting chlorophyll against heat stress were presented. A functional study on transgenic tobacco plants demonstrated the activity of NtHSP70-1 by the measurement of chlorophyll contents. Transgenic plants overexpressing NtHSP70-1 indicated less damaged chlorophyll contents, whereas the antisense constructs, which suppressed HSP70 expression, and control, containing only the vector, showed impaired chlorophyll contents. These results suggest that NtHSP70-1 plays an important role in thermotolerance in tobacco plants. Measurement of chlorophyll contents in the transgenic seedlings after heat stress was used as an indicator of thermotolerance. Transgenic tobacco seedlings constitutively overexpressing NtHSP70-1 showed increased chlorophyll contents compared with seedlings carrying only the vector and seedlings carrying the antisense constructs (Fig. 4, Table 2). This can be due to NtHSP70-1 protecting proteins involved in chlorophyll synthesis and thus inhibiting chlorophyll loss under heat-shock condition. Synthesis of chlorophyll from the precursor, glutamic acid, requires 15 or more enzymes [4] and activities of these enzymes will decide the level of chlorophyll accumulation in the seedlings. While chlorophyll accumulation is essential for normal development of seedlings, prevention of chlorophyll loss is also required. Chlorophyll loss is one of the major symptoms of leaf senescence, which is attributed to chlorophyll catabolism by oxygenase, an enzyme catalyzing chlorophyll breakdown [11]. Leaf senescence is the final stage of leaf developmental process that leads to death, limiting the life span or longevity of a leaf. Therefore, this study suggests that increased level of NtHSP70-1 in seedlings contribute to normal development and growth in plants after heat shock.

Chlorophylls, the pigments responsible for the characteristic green color of plants, are easily degraded during environmental stresses [13,31]. Severe stresses inactivates chlorophyllase and enzymes preventing senescence and rapid loss of green color and so induce damaged tissue to break chlorophyll [10,35]. Chlorophyll degradation results in dis-

coloration, which have been known to occur due to the conversion of chlorophylls to pheophytins. Therefore, chlorophyll retention has been used as a measure of health of plants under stresses [33]. In this study, seedlings overexpressing NtHSP70-1 indicated low level of discoloration after heat stress (Fig. 2, Fig. 3 and Table 1). This suggests that NtHSP70-1 prevents chlorophyll degradation under heat stress. From these results, it was demonstrated that increased level of NtHSP70-1 can contribute to normal development and growth of plants after heat shock. In plants, the major chlorophylls have been reported as chlorophyll *a* and *b*, which occur in the approximate ratio of 3:1 [37]. In addition, chlorophyll *a* was reported to be thermally less stable than chlorophyll *b*, which is derived from chlorophyll *a* [5,30,31,34]. Therefore, protection of chlorophyll *a* from stresses is important in plants. The results shown in Figure 3 supported the fact that NtHSP70-1 helps protect chlorophyll *a* in plants from heat stress and together with data in Table 2, it can be inferred that HSP70 chaperones confer a protective effect on chlorophyll *a* and *b* against heat stress.

According to reports, HSP70 protein is involved in resistance to photoinhibition: overexpression of HSP70 reduces photoinactivation of PSII and enhances recovery, whereas underexpression causes the opposite effect. HSP70 acts by both preventing the destruction of inactivated reaction centers and promoting the synthesis of new centers [27]. The repair of damaged PSII reaction centers is the most important means by which plant cells keep these centers functional during and after stress. This study did not identify whether NtHSP70-1 contributed to protection of PSII. Although these protective mechanisms by NtHSP70-1 were not presented, this work has produced the important finding of the association of HSP70 with chlorophyll.

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초록 : NtHSP70-1에 의한 클로로필의 고온 내성 효과

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고온 단백질 heat shcok protein 70 (HSP70)은 분자샤페론으로써 환경스트레스와 발달단계 동안 단백질을 보호하고 합성하는 다양한 과정에 관여하는 기본적인 단백질이다. 하지만 그 생물학적 기능이 식물에서 아직 정확하게 밝혀지지 않았다. 이에 본 연구에서는 담배에서 고온에 의해 유도된 HSP70인 NtHSP70-1 (AY372069)를 분리하여 그 기능을 연구하였다. NtHSP70-1의 고온 내성 기능을 분석하기 위해 NtHSP70-1이 식물 형질전환용 벡터인 pBKS1-1에 sense 또는 antisense 방향으로 도입되어 형질전환된 식물체와 pBKS1-1만 도입된 형질전환 식물체들을 제조하였다. 형질전환체에 있어서 NtHSP70-1의 발현량은 western blot 분석법을 사용하여 수행하였고 확인된 형질전환체들은 고온 내성 기능분석에 이용되었다. 그 결과 고온 환경에 있어서 NtHSP70-1이 과다발현된 형질전환체들은 그 클로로필의 함량과 생존율이 정상환경 일 때와 유사하였고 반대로 벡터 또는 벡터인 pBKS1-1에 antisense 방향으로 도입되어 형질전환된 식물체들은 클로로필의 파괴로 인한 감소된 생존율을 나타내었다. 고온 처리된 형질전환 식물체에서 클로로필의 함량비교 결과로 NtHSP70-1이 클로로필을 보호함으로써 식물의 고온내성에 기여함을 알 수 있었다.