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Abiotic tolerance of tobacco plants transformed with DnaK from a halotolerant cyanobacterium *Aphanothece halophytica*

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Introduction

The DnaK/HSP70 family is a molecular chaperone that binds non-native states of other proteins and assists them to reach a functional conformation. DnaK/Hsp70 proteins are induced by various environmental stresses and are believed to function in the protection and recovery of cells from the ill effects of stress. However, the critical factors conferring the enhanced temperature tolerance in higher plants are still poorly understood. Genetic engineering technology would be a useful approach for conferring thermotolerance in living organisms. Previously, we had isolated a *dnaK1* gene from the halotolerant cyanobacterium *Aphanothece halophytica* (*A. halophytica*) which can grow in high salinity conditions of up to 3 M NaCl (Lee et al. 1997). The *dnaK1* gene encodes a polypeptide (DnaK1) of 721 amino acids which contains a longer C-terminal segment (70 to 90 extra amino acid residues) than other DnaK/Hsp70 family members. The DnaK1 from *A. halophytica* was shown to exhibit extremely high protein folding activity under high salinity conditions (Hibino et al. 1999). Transformation of tobacco with the DnaK1 from *A. halophytica* was shown to enhance the salt tolerance of tobacco (Sugino et al. 1999) Therefore, we examined whether the overexpression of DnaK1 from a halotolerant cyanobacterium *A. halophytica* could confer tolerance to high-temperature stress (Ono et al. 2001).

Materials and methods

For the transformation of tobacco plant, the *dnaK1* gene from *A. halophytica* was ligated into the *Xba*I/*BamH*I site of the binary vector pBI121 in which the glucuronidase gene was removed and was placed between the cauliflower mosaic virus 35S (35S CaMV) promoter and nopaline synthase terminator (Nos-ter) regions. The plasmid was introduced into *Nicotiana tabacum* by the *Agrobacterium*-mediated method (Sugino et al. 1999). The standard procedure for germination was as follows. Seeds were surface sterilized with 2.5% NaClO and 0.002% Triton X-100 for 5 min, and then placed in petri dishes that contained Murashige & Skoog agar medium (0.8% agar). The plates were incubated in a growth chamber with 16-h light (200 $\mu E\, m^{-2}s^{-1}$) 27°C /8-h dark at 25°C under 60% relative humidity. High temperature treatment during the germination was carried out with the incubation at 40°C during the light period (16h) and 25°C during the dark period (8h). For high temperature treatment, seeds were incubated with water for 6h at various temperatures in the

dark. For the measurement of plant growth, we took photographs with an optical microscopy (Olympus IX70) and analyzed them with Fluoview software (Olympus). The quantum yield in PSII under illumination, (Fm'-F)/Fm', was measured with a Mini-PAM Chl fluorescence system (Walz, Germany) using actinic light of 30 $\mu E\, m^{-2}s^{-1}$ and a 800-ms pulse length of saturating white light of 1500 $\mu E\, m^{-2}s^{-1}$. Here, F and Fm' represent the fluorescence levels under irradiation before and after a saturating flash, respectively. CO_2 fixation and stomatal transpiration were measured with a portable photosynthesis system HCM-1000 (Walz, Germany). The relative water contents were calculated by the equation, (Fw-Dw)/(Tw-Dw), where, Fw, Dw, and Tw represent the fresh weight, dry weight, and turger weight, respectively. The Tw was the weight of sample after overnight incubation with water in the dark. Protein extraction, SDS-PAGE, Western blottings were carried out as described (Wang et al., 1999, Ono et al., 2001).

Results

We examined the effect of high temperature during the germination of tobacco. Under normal conditions, both the control (Fig. 1A) and transformant (Fig. 1C) began to germinate 3 days after imbibition and about 95% of seeds had germinated after 5 days. In contrast, when the temperature of the light period was increased to 40°C, germination was observed 4 days after imbibition in both the control and transformant seeds. Only 27% of the control tobacco seeds germinated (Fig. 1B), whereas 82% of the transgenic tobacco seeds (Fig. 1D) germinated. These results indicate that the overexpression of DnaK confers thermotolerance during the germination phase.

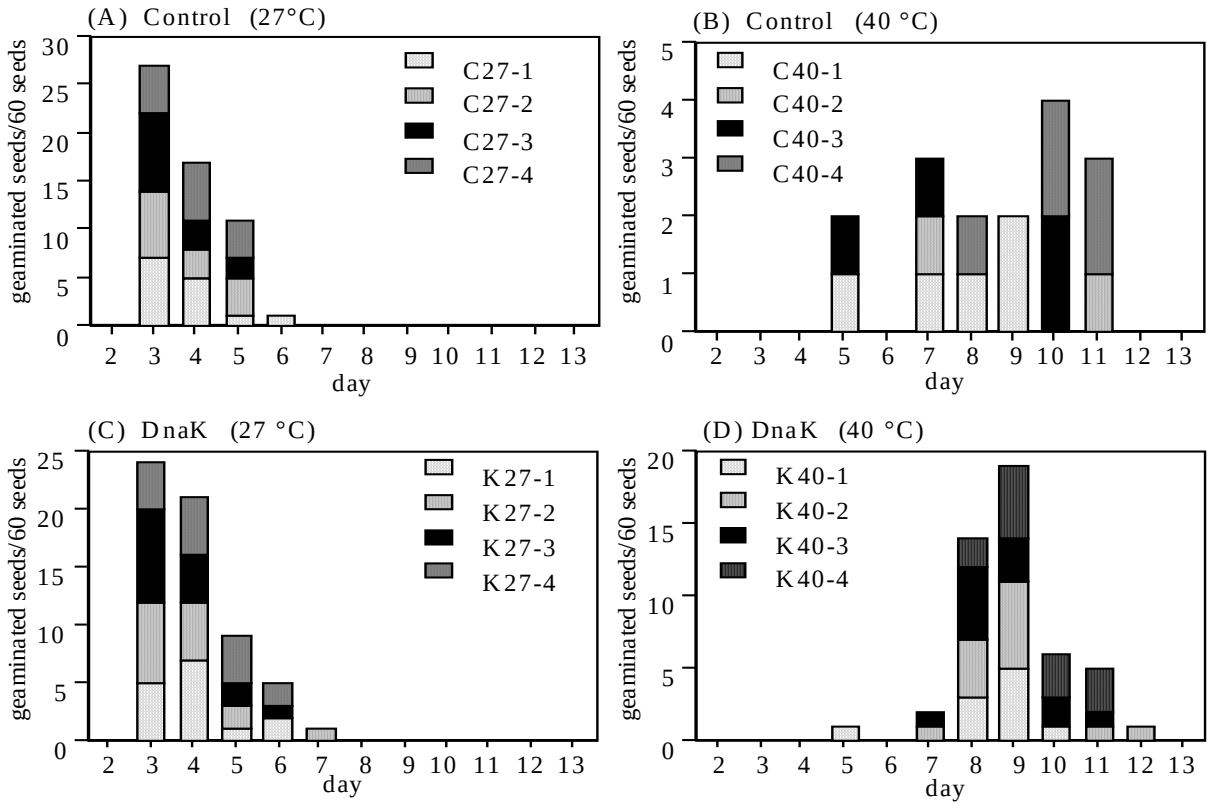

Fig. 1. Effects of high temperature on the germination of wild-type and transformant seeds.

Next, we examined the effect of high temperature during imbibition on the germination of tobacco plants. Dry seeds were imbibed at 40, 45, 50, and 55°C for 6h in the dark. Then seeds were placed on agar medium under the normal conditions (16h light at 27°C and 8h dark at 25°C). When the imbibition was carried out at 25°C, the subsequent germination patterns were almost the same in the control and transformant plants. In contrast, the imbibition at 40-50°C delayed the subsequent germination in both the control and transformant plants. However, the germination was more delayed in the control plants. The imbibition at 55°C completely inhibited the germination in both the control and transformant plants.

The effects of high temperature on seedling growth were also examined. When the root extended to 5 mm, about 6 days after imbibition, the control and transformant plants were treated at 40°C during the light period for 1 day and then incubated 2 days under normal conditions. This treatment reduced the subsequent growth rate in both plants. However, the reduction was more pronounced in the control plants than in the transformants.

The effect of high temperature on the mature plants was also examined. Six-week-old plants were exposed to high temperature (40°C). The response to the high-temperature treatment differed between the young expanding leaves and mature leaves. In young leaves, the quantum yields of electron transport in the control plants decreased whereas those of the transgenic plants were retained almost at the pre-treatment levels. In contrast, the quantum yields of electron transport in the mature leaves decreased at almost the same rate although the start of the decrease was later in the transgenic plants. Similar effects were observed for CO_2 fixation rates. The relative water contents in the control and transformant were similar before heat stress. Upon heat stress, the relative water contents of both plants decreased, but more significantly in the control plants. All results indicate that the accumulation of DnaK1 prevents the inhibition of plant growth and photosynthesis in the young seedlings.

The transgenic and control tobacco seeds were extracted and immunoblotting was carried out to investigate the expression of the *dnaK1* gene. The antibody against DnaK1 from *A. halophytica* cross-reacted with the seed extracts from the transgenic tobacco although its intensity was low and the levels increased during germination. The levels of DnaK1 in the transgenic plants were not altered by high-temperature. We examined whether or not the expression of tobacco Hsp/Hsc70 proteins was affected by the expression of DnaK1. Hsp/Hsc70 proteins were detected in the germinating seeds as well as in young seedlings under normal conditions. Although their levels were increased upon high-temperature treatment, the increase was similar in the control and transformant plants. These results suggest that the expression of tobacco Hsps was not affected by the expression of DnaK1.

Discussion

The present study demonstrated that DnaK1 from a halotolerant cyanobacterium *A. halophytica* could be expressed in tobacco plants, thereby conferring the tobacco plant resistance to high-temperature stress to the tobacco plant. The enhancement of thermotolerance was clearly observed in actively developing tissues such as germinating seeds, emerging shoots , and leaves. In contrast, in the mature leaves, the photosynthetic activity decreased almost at the same rate in control and transformant although a lag was observed in the transformant. These findings suggest that actively expanding cells might be more sensitive to high-temperature stress than the mature cells. DnaK1 might protect against the inhibition of protein synthesis, transcription, and replication of DNA at high temperature rather than the inactivation of already synthesized and assembled proteins. Delay of decrease of photosynthesis in mature leaves suggests that the pre-existence of DnaK1 prevents the inhibition of photosynthesis due to high-temperature stress, but is insufficient after the onset

of expression of tobacco Hsps. It was also suggested that the pre-existing DnaK1 protects the reversible inhibition of imbibition and germination due to high-temperature stress. It might be worthwhile to note that high temperature treatment applied daily in this study is different from those previously used. In previous studies, high temperature treatment for a short time (within 1h) was applied. The significance of a protective role of DnaK during a long time high temperature treatment remains to be evaluated.

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