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Original Paper

Overexpression of DnaK chaperone from a halotolerant cyanobacterium *Aphanothece halophytica* increases seed yield in rice and tobacco

Akio Uchida[1,6], Takashi Hibino[2], Takiko Shimada[3], Masahiko Saigusa[4], Tetsuko Takabe[1], Etsuko Araki[5], Hiroshi Kajita[6], Teruhiro Takabe[2,5,*]

[1] Graduate School of Agricultural Science, Nagoya University, Nagoya 464-8601, Japan; [2] Graduate School of Environmental and Human Sciences, Meijo University, Nagoya 468-8502, Japan; [3] Research Institute of Agricultural Resources, Ishikawa Agricultural Collage, Ishikawa 921-8836, Japan; [4] Graduate School of Agricultural Science, Tohoku University, Sendai 981-8555, Japan; [5] Research Institute, Meijo University, Nagoya 468-8502, Japan; [6] SCIVAX Co., Kawasaki, 213-0012, Japan

* E-mail: takabe@ccmfs.meijo-u.ac.jp Tel: +81-52-838-2277 Fax: +81-52-832-1545

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Abstract The DnaK/Hsp70 family is a molecular chaperone that binds non-native states of other proteins, and concerns to various physiological processes in the bacterial, plant and animal cells. Transgenic tobacco plants expressing a molecular chaperone DnaK from a halotolerant cyanobacterium Aphanothece halophytica show enhanced seed yields as well as enhanced tolerances for salt and heat stresses. High-temperature treatment during the reproductive stage decreased total dry weight of seeds in both transgenic and wild-type tobacco, but more severely in the wild-type. Transgenic tobacco plants exhibited higher activities of ascorbate peroxidase and catalase than wild-type plants. Similar results were obtained for salt stress during the reproductive stage. Transgenic rice plants expressing ApDnaK was also constructed. Transgenic rice plants exhibited the enhanced activities for Calvin-cycle enzymes, and showed faster growth and higher seed yield compared with the wild-type rice under normal growth conditions. Transgenic rice plants also showed enhanced tolerance for high temperature and salt stress compared with the wild-type rice. These results suggest a relation of increased folding activity with enhanced stress tolerance, increased seed yield, and total plant biomass.

Key words: *Aphanothece halophytica*, chaperone, DnaK, high temperature stress, rice, salt tolerance, seed yield.

Salt, drought, and heat stresses are often combined in nature. Much research is aimed toward the breeding of crop cultivars with improved salt and heat tolerances. Organisms that thrive in harsh environments must possess specific mechanisms for adapting to stressful environments. One such mechanism would be the induction of molecular chaperones, heat shock proteins (HSPs), which comprise several evolutionarily conserved protein families such as Hsp100 (ClpB), Hsp90, Hsp70 (DnaK), Hsp60 (GroEL), and small Hsp (prokaryotic chaperones in parentheses) (Boston et al. 1996; Hart 1996; Bukau and Horwich 1998). Extensive studies suggest that different chaperones follow distinct strategies to prevent protein misfolding and aggregation in the highly crowded cellular environment. For example, the monomeric Hsp70 recognizes short hydrophobic peptide segments and binds at an early stage of folding (Hart 1996; Bukau et al. 2000). The oligomeric Hsp60 forms double ring structure capable of enclosing large domain of proteins to promote the later stage of folding. Small Hsps are involved in trapping unfolded proteins (Boston et al. 1996; Lee et al. 1997b) which are sequentially refolded by Hsp70 and then by Hsp60 (Veinger et al. 1998). Similarly, aggregated proteins can be resolubilized and refolded by the sequential action of Hsp100 and Hsp70 (Glover and Lindquist 1998). Hsp90 is involved in activation of many signal transduction proteins (Buchner 1999; Young et al. 2001).

In spite of HSP's function, the positive role of HSPs for thermo- and salt-tolerances in whole plants has been demonstrated in limited examples. Although the overexpressions of Hsp70 from *Drosophila* and mammalian conferred the thermotolerance of their organisms (Solomon et al. 1991; Kim et al. 1995), the overexpression of small Hsp from soybean was not sufficient to confer the thermotolerance in transgenic tobacco plants (Schoffl et al. 1987). Later, the transgenic plants constitutively expressing the heat shock factor

Abbreviations: Ap, Aphanothece halophytica; CaMV, cauliflower mosaic virus; HSF, heat shock factor; Hsp, heat shock proteins; MS, Murashige and Skoog; PRK, phosphoribulokinase; FBPase, cytosolic fructose-1,6-bisphosphatase; SPS, sucrose phosphate synthetase.

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(HSF) of HSP was shown to confer the thermotolerance (Lee et al. 1995). The overexpressions of carrot small Hsp (Malik et al. 1999) and *Arabidopsis* Hsp101 (Queitsch et al. 2000; Hong and Vierling 2001) have shown to enhance the thermotolerance of their own cells. The overexpression of *Arabidopsis* small Hsp was shown to enhance the osmotolerance of *Arabidopsis* (Sun et al. 2001). We also showed that the overexpression of DnaK from a halotolerant cyanobacterium *Aphanothece halophytica* (ApDnaK) which was isolated from The Dead Sea, conferred the tolerances for salt (Sugino et al. 1999) and high temperature (Ono et al. 2001) in transgenic tobacco plants. However, all these studies did not show the effects of overexpression of HSP on crop yield as well as growth promotion. Crop yield is one of the most important factors for agriculture. ApDnaK was previously shown to exhibit extremely high protein folding activity even under high salinity conditions (Hibino et al. 1999). Here, we report that the transgenic rice and tobacco plants expressing ApDnaK could increase the seed yield and plant biomass in the laboratory conditions.

Materials and methods

Construction of vector and transformation

Tobacco plant (*Nicotiana tabacum* cv. Petit Havana SR1) was transformed with the *dnaK* gene from *A. halophytica* as described previously (Sugino et al. 1999). Four transgenic lines with vector alone (C1-C4) and with *ApdnaK* gene (T15, 28, 36, and T48) were used. For the transformation of rice plant (*Oryza sativa* L. cv. Notohikari), the *ApdnaK* gene was ligated into the *Nco*I site of the binary vector pCAMBIA1301 which located downstream of the CaMV 35S promoter. The plasmids were introduced into rice by the *Agrobacterium*-mediated method. Plants were grown aseptically on Murashige & Skoog (MS) (Murashige and Skoog 1962) agar medium supplemented with 3% (w/v) sucrose containing 50 μg/ml antibiotics. Kanamycin and hygromycin were used for the selection of transformants of tobacco and rice, respectively. Then, seedlings were grown hydroponically in 10-fold-diluted MS medium in a growth chamber (Sanyo MLR-350HT, Japan) with indicated light (16 h at 200 $\mu Em^{-2} s^{-1}$)/dark (8 h) cycle. Twenty-five transgenic rice lines were analyzed.

Plant materials

To investigate stress tolerance in the reproductive stage, seeds of tobacco were sown onto Petri dishes and germinated for 4 weeks and the plants were grown on soil in plastic pots and grown in a growth chamber (16 h light period, 27°C, humidity 70%; 8 h dark period, 25°C, humidity 70%). The irradiance at leaf level was 200 $\mu mol\, m^{-2} s^{-1}$. After growing for about 14 weeks, the flower buds started to emerge. For high temperature treatment, the growth temperature during the light period was changed as indicated, but that of the dark period was kept at 25°C. For salt stress, plants were grown in fresh 10-fold diluted MS medium containing the indicated concentrations of NaCl under the same day/night conditions. Photographs of the plants were taken with an optical microscopy (Olympus IX70) and analyzed them with Fluoview software (Olympus).

To investigate photosynthetic activity, enzymes activity, soluble sugar contents, and osmolarity in rice, seeds of rice were sown onto Petri dishes for 4 days and the seedlings were grown on soil in plastic pots and grown in a growth chamber (16 h light period, 27°C, humidity 70%; 8 h dark period, 25°C, humidity 70%). To investigate the productivity of rice seed, seeds of rice were sown onto Petri dishes for 4 days and the seedlings were grown on soil in plastic pots (volume 300 ml) for 4 weeks, and then were transferred in plastic pot (volume 3 liter) and grown in growth chamber (10 h light period, 27°C, humidity 70%; 14 h dark period, 25°C, humidity 70%). The irradiance at leaf level was 400 $\mu mol\, m^{-2} s^{-1}$. For salt stress, rice plants were grown on soil containing 10-fold diluted MS medium and the indicated concentrations of NaCl under the same day/night conditions.

Measurement of growth parameters

Net CO_2 fixation activity was measured with the HCM-1000 portable photosynthesis system (Heinz Walz GmbH, Germany). The osmolarity of leaves was measured by the vapor pressure method (model 5520; Wescor, Logen, UT, USA). The dry weights (DW) of plants were measured after drying at 70°C. Harvest index was calculated as (seeds DW/(seeds DW+ aerial parts DW))×100.

Activities of active oxygen quenching enzymes

Activities of active oxygen quenching enzymes were measured as previously described (Tanaka et al. 1999). Leaf tissue was homogenized in extraction buffer (50 mM potassium phosphate (pH 7.8), 1 mM EDTA, 5 mM ascorbic acid, and 0.2% Triton X-100) (Tanaka et al. 1999). The homogenate was centrifuged at 20,000×g for 10 min at 4 °C, and the supernatant was used for antioxidant enzyme assays. The ascorbate peroxidase (APX) activity in the extract was determined spectrophotometrically by measuring the oxidation of ascorbate at 290 nm. The assay mixture contained 50 mM potassium phosphate (pH 7.0), 0.2 mM H_2O_2 and 0.2 mM ascorbate. The activity of glutathione reductase (GR) was measured by monitoring the oxidation of NADPH at 340 nm. Reaction mixtures contained 50 mM potassium phosphate (pH 7.5), 1 mM EDTA, 0.5 mM oxidized glutathione and 50 μM NADPH. Catalase activity was assayed by monitoring the consumption of H_2O_2 at 240 nm in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7.0) and 2 mM H_2O_2. All spectrophotometric assays were carried out at 25°C.

Determination of sugars and starch contents in plants

Leaves were frozen with liquid nitrogen, extracted with boiling 80% (v/v) ethanol, and centrifuged at 10,000×g for 10 min at 4°C. The extraction was repeated one more. The total soluble sugar in supernatant was determined with the anthrone reagent (Roe 1955). For the extraction of starch, the pellet from the soluble sugar extract was suspended in 0.2 M KOH and incubated in boiling water for 30 min. After adjusting pH to 5.5 by sodium acetate buffer, the samples were digested with amyloglucosidase at 37°C for 16 h (Goldschmidt and Huber 1992). Starch content was calculated from the resulting soluble sugar contents. For the quantification of fructose, glucose, and

sucrose, the alcoholic extracts were dried up and dissolved in sterilized water. The individual sugars were determined using a Shimadzu HPLC system for sugar analysis.

Enzyme activities for Calvin-cycle and sucrose biosynthesis

Leaves were homogenized with a buffer A (50 mM 4-morpholinepropanesulphonic acid (MOPS)-KOH, pH 7.3, 5 mM $MgCl_2$, 1 mM EDTA, 16 mM mercaptoethanol, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol), 2 mM benzamidine, 1 mg/l leupeptin, and 2 mM phenylmethylsulfonyl fluoride. The crude extracts were desalted through Sephadex-G25 column equilibrated with the buffer A, and used for enzyme assays. Phosphoribulokinase (PRK) activity was assayed as described (Miyagawa et al. 2001). Cytosolic fructose-1,6-bisphosphatase (FBPase) was separated from the chloroplastic isoform by ion-exchange chromatography, and their FBPase activities were assayed as described (Miyagawa et al. 2001). Sucrose phosphate synthetase (SPS) activities were assayed under V_{max} condition as described (Huber et al. 1991).

Detection of mRNA and proteins

Leaf tissue was homogenized in extraction buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Total protein of the homogenizate was determined by the method of Lowry et al. as previously described (Lee et al. 1997a). Proteins (50 μg per sample) were separated on SDS-PAGE with 12.5% acrylamide according to Laemmli as previously described (Hibino et al. 1999). Immunoblotting analysis was done as previously described (Sugino et al. 1999). An antiserum raised against the *A. halophytica dnaK* was prepared as described (Hibino et al. 1999). Northern blotting was carried out using a *dnaK* gene specific probe as previously described (Lee et al. 1997a).

Statistical analyses

Statistical analyses were carried out using ANOVA and Bonferroni-type t-test for determination of significant differences.

Results

Overexpression of ApDnaK enhanced seed yields in tobacco under high temperature and salt-stress conditions

We examined the effect of high temperature during the reproductive stage of tobacco. Under normal conditions (16 h light at 27°C and 8 h dark at 25°C), both the wild-type and transgenic tobacco plants grew at similar rates (data not shown). When the flower buds started to emerge, temperature during light period was increased to 33 or 37°C, although dark-period-temperature was the same. After two to three weeks later, the ApDnak-tobacco plants were phenotypically distinguishable from the wild-type plants (Figure 1A). Number of flowers was slightly higher in wild-type tobacco than that of the transgenic plants when plants were grown at 27°C during light period (Figure 1B). Their numbers were similar when plants were irradiated at 33°C, but significantly increased at 37°C. The ratio of flowering number

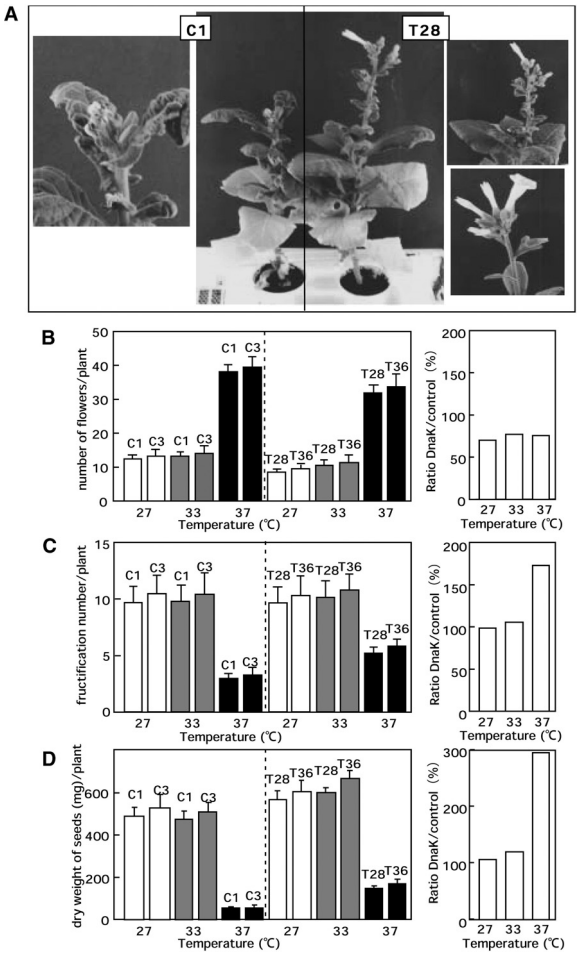

Figure 1. Effects of high temperature during the reproductive stage of the wild-type and transgenic tobacco plants. When the flower buds started to emerge (about 5 months after germination), temperature of light period was changed (16 h light at 33 or 37°C and 8 h dark at 25°C). Then, numbers of flowers and fructifications, and dry seed weight were measured. (A) Photographs of tobacco plants. (B) Effects of high temperature on the number of flowers per plant. (C) Fructification number per plant. (D) Dry seed weight per plant. C1 and C3, control transgenic plant with vecor alone. T28 and T36 are transgenic plants expressing ApDnaK. Data of (B), (C), and (D) are presented as mean values±SD calculated from three sets of independent experiments.

between the wild-type and transgenic plants was almost the same regardless of irradiation temperatures (Figure 1B, right panel). Fructification numbers were similar between the wild-type and transgenic plants when the irradiation temperatures were 27 or 33°C (Figure 1C). When the irradiation temperature was increased to 37°C, the fructification number significantly decreased in both the wild-type and transgenic plants, but more severely decreased in the wild-type plants (Figure 1C). Therefore, the fructification number of transgenic plants was about 1.8-fold larger than the wild-type plants. Crop yield depends on seed number and seed weight. As shown in Figure 1D, dry weight of seeds in the transgenic plants was slightly larger compared with wild-type when the irradiation temperature was 27 or 33°C. When the

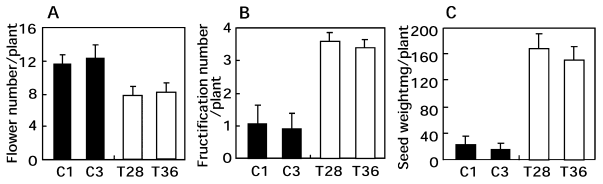

Figure 2. Effects of NaCl (0.6 M) during the reproductive stage of the wild-type and transformant tobacco plants. (A) Number of flowers per plant. (B) Fructification number per plant. (C) Dry seed weight per plant. C1 and C3, control transgenic plant with vecor alone. T28 and T36 are transgenic plants expressing ApDnaK. Data are presented as mean values±SD calculated from three sets of independent experiments.

irradiation temperature was increased to 37°C, dry weight of seeds decreased in both plants. However, the decrease was more severe in the wild-type and consequently seed yields in the transgenic plants were three-fold higher than those in wild-type plants. These results indicate that the overexpression of ApDnaK alleviated the decrease of seed yields in tobacco due to high temperature stress. It should be noted that high temperature during light period, 37°C, occurs in many places of world including summer at Nagoya in Japan.

We also examined the effects of salt stress on the seed yield of tobacco plants. When the flower buds started to emerge, the wild-type and transgenic tobacco plants were imposed to salt stress (0.6 M NaCl) without changing the light conditions (16 h light at 27°C and 8 h dark at 25°C). As shown in Figure 2A, numbers of flowers in the wild-type and transformant per plant were about 12 and 8, respectively which were almost the same as those in non-stressed plants (Figure 1B). By contrast, fructification number and dry weight of seeds significantly decreased under salt-stress conditions in both wild-type and transgenic plants, but more severely in the wild-type plants (Figures 2B and 2C). Consequently, the fructification number and dry weight of seeds in transgenic plants were approximately 3.4- and 6-fold larger than those of the wild-type plants, respectively.

Transgenic tobacco plants exhibited higher activities of ascorbate peroxidase and catalase than wild-type plants

We examined several parameters in the leaves of reproductive stage of wild-type and transgenic tobacco plants which were irradiated at high temperature. The levels of chlorophyll, soluble protein, and auxin in upper leaves were similar between wild-type and transgenic tobacco plants (data not shown). Among the active oxygen quenching systems, the total activities of superoxide dismutase (SOD) and glutathione reductase (GR) were also similar between wild-type and transgenic plants (Figures 3B and 3D). By contrast, the total activities of APX and catalase were significantly higher in transgenic plants than in wild-type plants when the irradiation temperature was 37°C (Figures 3A and 3C). These results indicate the importance of active oxygen quenching systems for crop yield at high temperature.

Overexpression of ApDnaK enhanced seed yield in rice

Rice plants were transformed with the same *ApdnaK* gene by the *Agrobacterium*-mediated method (Figure 4A). Four homozygous T_3 transformants (D1, D3 D15, and D27) were used for growth experiments (Figsures 4B and 4C). Transgenic plants were phenotypically distinguishable from the wild-type plants when rice plants were grown even under the conditions of 14 h light at 28°C and 10 h dark at 23°C (Figure 4D). The transgenic plants, except for D27, grew faster and bigger than wild-type plants (Figure 4D). Especially, D1 and D3 plants were about 1.4- and 1.5-fold larger in height compared with the wild-type plants. Western blot analysis showed the accumulation of ApDnaK in the transgenic plants, but not in wild-type plants (Figure 4C). These results indicate that the overexpression of ApDnaK protein enhanced the size of rice plant.

Next, we examined the crop yield of rice plants which were grown under the same growth conditions described above. It was found that the number of tiller of transgenic rice (D3) was already higher than that of wild-type rice at 60 days after sowing (Figure 5A). At 150 days after sowing, number of tiller of D1 and D3 was about 1.3-fold higher than that in the wild-type rice (Figure 5B). Dry weights of aerial parts, root, and seeds per plants in D1 and D3 were also larger than those of the wild-type rice, respectively (Figures 5C–E). Consequently, higher harvest index was obtained for D1 and D3 rice plants (Figure 5F). The rice plants (D1, D3, and D15) had more ears compared with wild type plants (data not shown). These results indicate that the overexpression of ApDnaK enhanced crop yield of rice plants.

Overexpression of ApDnaK in rice enhanced the enzyme activities for Calvin-cycle and sucrose biosynthesis

To clarify the reason for enhanced growth and seed yield in the transgenic rice plants, we measured photosynthetic activity. The net uptake of CO_2 in the 4-week-old leaf was higher than that of the wild-type plants at irradiances above $400\,\mu mol\,m^{-2}\,s^{-1}$ (Figure 6A). At irradiances $1{,}800\,\mu mol\,m^{-2}\,s^{-1}$, the transgenic rice exhibited 1.2-fold higher activity than that of wild-type plant (Figure 6A). These data suggest larger accumulation of carbohydrates in transgenic plants. Therefore, we measured the osmolarity. On histogram analyses of 2-week-old rice plants, ApDnaK plants (D3) exhibited significantly higher osmolarity compared with the wild-type plants (Figure 6B). Transverse section of

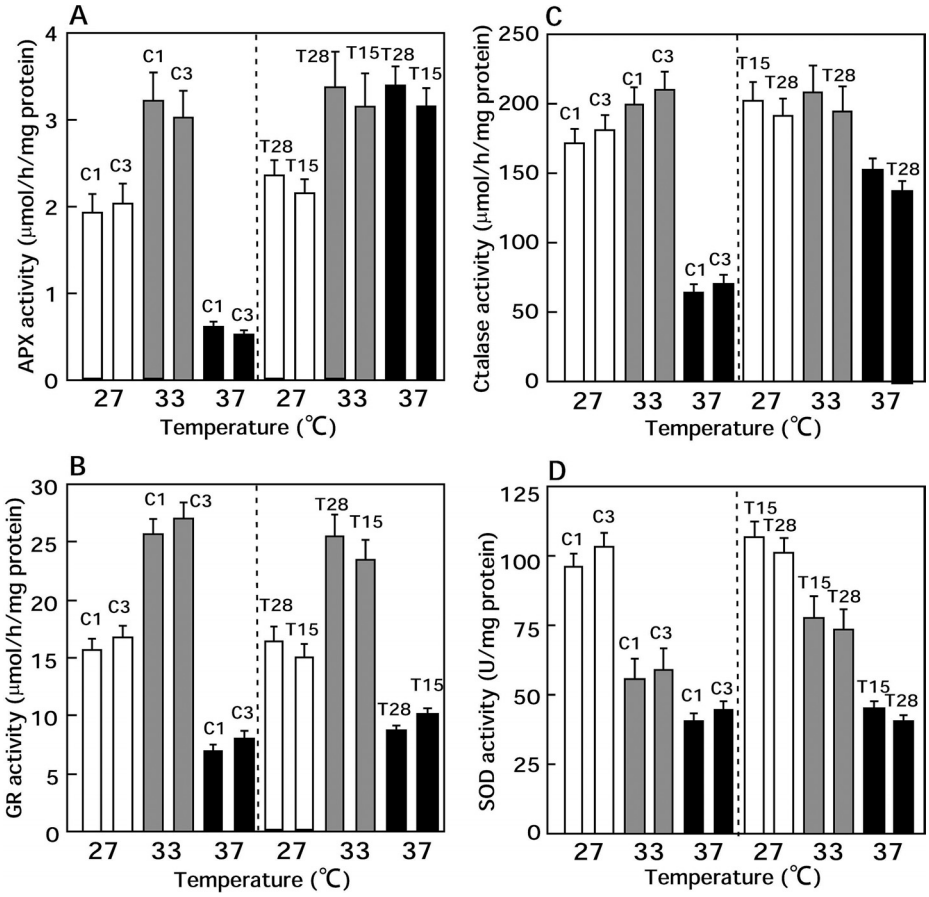

Figure 3. Activities of active oxygen quenching enzymes. Activities of active oxygen quenching enzymes were measured as described in Materials and methods. (A) Ascorbate peroxidase (APX) activity. (B) Glutathione reductase (GR) activity. (C) Catalase activity. (D) Superoxide dismutase (SOD) activity. C1 and C3, control transgenic plant with vecor alone. T15 and T28 are transgenic plants expressing ApDnaK. Data are presented as mean values±SD calculated from three sets of independent experiments.

first sheath and forth leaf exhibited larger size of phloem and leaf vein in D3 plants than that of the wild-type plants (Figure 6C). The contents of sucrose, starch, fructose, and glucose in D1 and D3 plant were higher than those in wild-type plant, respectively (Figure 6D). The enzyme activities of phosphoribulokinase (PRK) and plastidic FBPase, which are key enzymes in Calvin-cycle, and cytosolic FBPase and sucrose phosphate synthase (SPS), which are key enzymes in sucrose synthesis, in D1 and D3 rice were also higher than those in wild-type plant when plants were irradiated. No difference was observed when the plants were kept in dark. These data suggest that overexpression of ApDnaK enhanced photosynthesis and activities of Calvin cycle, and increased the sugar contents.

Overexpression of ApDnaK enhanced seed yields in rice under high temperature and salt-stress conditions

Next, we examined whether the overexpression of ApDnaK in rice plants enhances salt stress tolerance. To avoid ambiguity caused by different growth rates between the wild-type and transgenic plants, we used the rice plants with the same size. Five to six week-old rice plants were grown on soil under various salt-stress conditions for 4 weeks. Figure 7A shows the effects of NaCl on the fresh weight of aerial parts. The fresh weight of wild-type rice decreased with increasing the concentrations of NaCl (Figure 7A). Interestingly, the fresh weight of D1 and D3 rice plants increased with increasing the concentrations of NaCl, reached the maximum at 20 mM NaCl, and then decreased. When three to four month-old rice plants were grown on soil under various salt-stress conditions for 2 months, the dry weight of seeds in wild-type rice decreased with increasing the concentrations of NaCl (Figure 7B) whereas those of transgenic rice increased with increasing the concentrations of NaCl, reached the maximum at 20–50 mM NaCl, and then decreased. The seed weight in D1 and D3 was always higher than that of wild-type rice over the range of 0–150 mM NaCl. Similar results were observed for other parameters such as fresh weight, sugar, starch content, and SPS activity, phosphoriblokinase activity, and FBPase activity. These data indicate that the overexpression of ApDnaK enhanced the salt tolerance of rice plants at

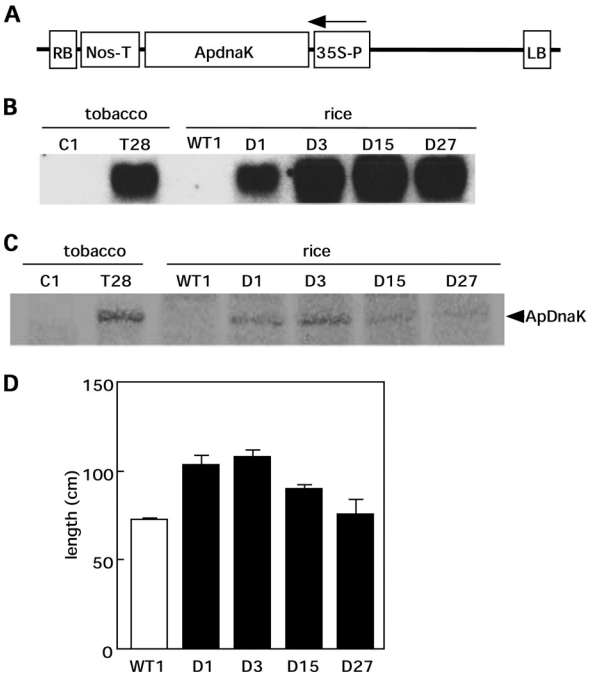

Figure 4. Construction of transgenic rice and their growth. (A) Binary vector. (B) Northern blotting. RNA was extracted from rice leaves and analyzed by using a *dnaK* specific probe. (C) Western blotting. Total proteins were extracted and analyzed by the immunoblotting technique with the antibodies raised against ApDnaK. (D) Plant height. Data of (D) are presented as mean values±SD calculated from three sets of independent experiments.

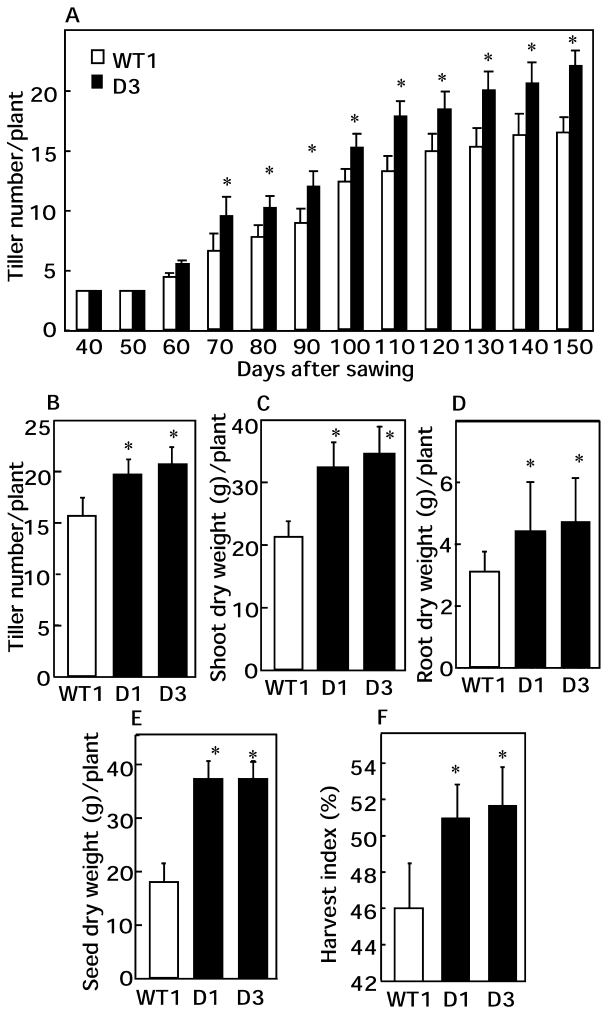

Figure 5. Number of tiller, plant weight, and seeds weight in wild-type and ApDnaK-rice plants. (A) Changes of tiller number. (B) Tiller number. (C) Dry weight of aerial parts. (D) Dry weight of root. (E) Dry weight of seeds. (F) Harvest index. The values in (B)–(F) were measured after growing for 150 days. Values are the means of ten replicates ±SD. The difference in the mean values between the wild-type and ApDnaK rice plants was found to be statistically significant (* $P<0.01$).

developmental and reproductive stages.

High temperature stress tolerance was also examined. When the leaves extended to 5.5 mm, temperature of light period was increased (16 h light at 42°C and 8 h dark at 25°C). Figure 8A shows the photograph of plants treated at a high temperature for 7 days. It can be seen that not only plant height, but also the root number and length were significantly larger in the transgenic rice plants compared with the wild-type. Figure 8B shows the effects of high temperature on plant elongation. After transfer to a high temperature, wild-type rice plants still grow during three days, but then the plant length did not increase more. By contrast, the transgenic plants continued to increase in plant height. The accumulation levels of ApDnaK also increased in the transgenic rice irradiated at high temperature (Figure 8C).

Discussion

The data presented in the above clearly indicate that transgenic tobacco and rice plants expressing ApDnaK which was targeted to cytosol produced more seeds than wild-type plants and conferred the tolerances for salt and heat stresses (Figures 1, 2, 7, and 8). Although several transgenic plants have been produced to investigate the potential importance of HSPs for the abiotic stress tolerances (Lee et al. 1995; Malik et al. 1999; Sugino et al. 1999; Queitsch et al. 2000; Hong and Vierling 2001; Ono et al. 2001; Sun et al. 2001), the effects on crop yields have not been investigated previously. Flowering and seed formation are very sensitive to abiotic stresses. Increase of temperature during light period from 27 to 37°C drastically altered number of flowers (Figure 1B) and fructifications (Figure 1C) and dry weight of seeds (Figure 1D). But, at 37°C, the dry weight of seeds and fructification number in transgenic tobacco plants were about 3.0- and 1.8-fold larger than those in wild-type plants. Similar results were observed for salt stress (Figure 2). It was found that the levels of chlorophyll, soluble protein, auxin, superoxide dismutase did not change so much between the wild-type and transgenic tobacco plants, but the activities of APX and catalase were significantly higher in transgenic plants than that in

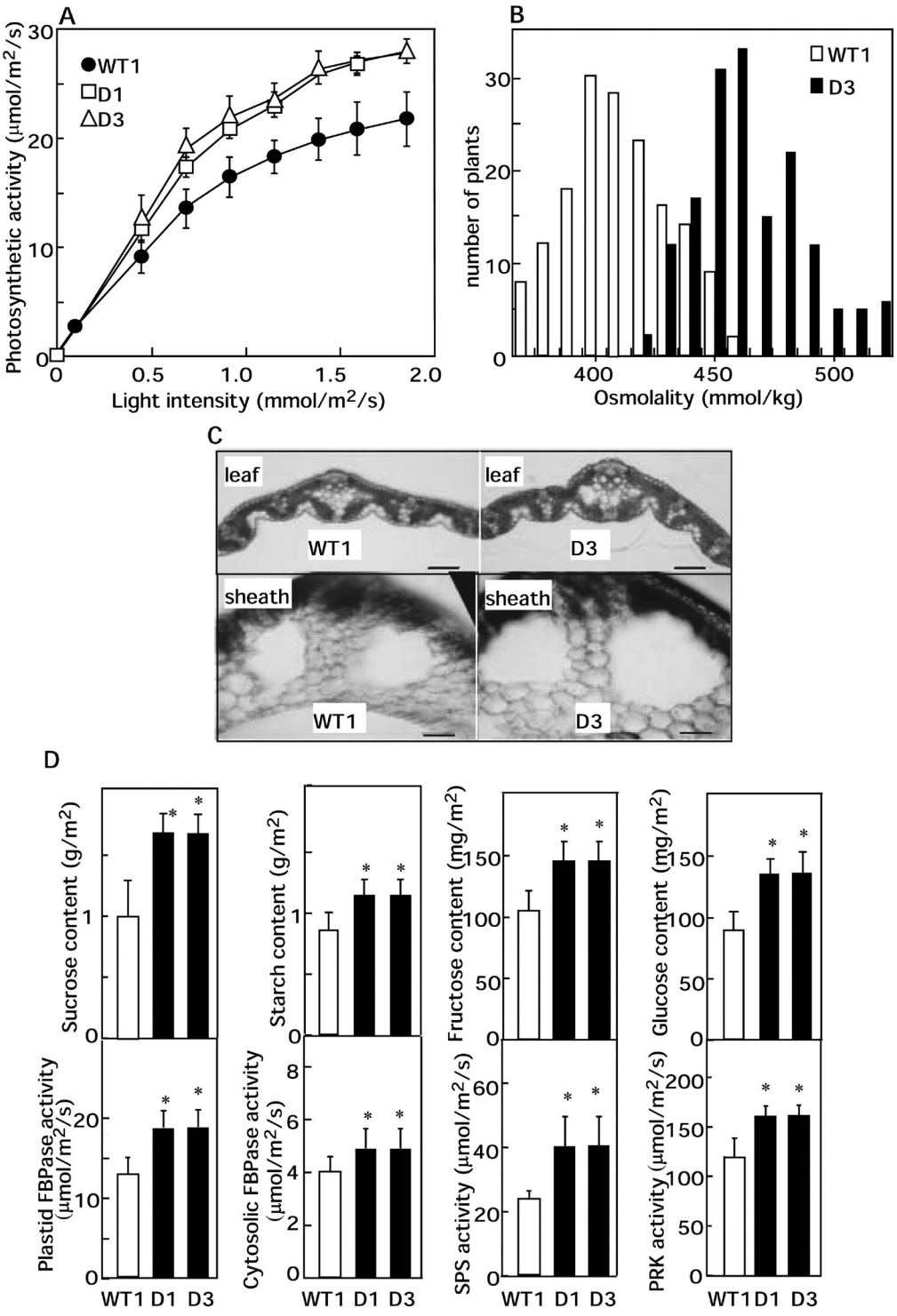

Figure 6. Various parameters related to photosynthesis. Rice plants were grown on soil for 14 days. (A) Net CO_2 uptake activity. For the measurement, six plants were used. (B) Histograms of osmolality. About one hundred and fifty plants were used for the analysis. (C) Transverse section of first sheath and forth leaf on the wild-type and ApDnaK rice plants. Rice seedlings were grown on soil under non-stress conditions for 28 days. Transverse section of first sheath and forth leaf on the wild-type and ApDnaK rice plants were measured. Bar is 100 μm. (D) Enzyme activity and sugar contents. Rice plants were grown for 3 weeks. Carbohydrates content and enzyme activities involved in Calvin-cycle and sucrose biosynthesis in the wild-type and ApDnaK rice plants are shown. Values are the means of seven replicates ±SD. The difference in the mean values between the wild-type and ApDnaK rice plants was found to be statistically significant (* $P<0.01$).

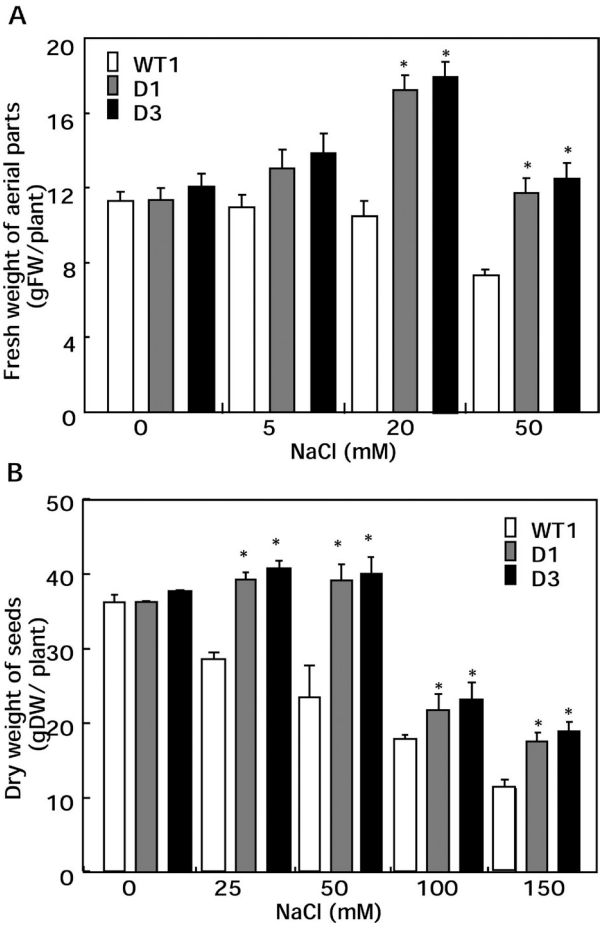

Figure 7. Effects of salt stress on the growth and seeds yields. (A) Fresh weight of aerial parts. (B) Dry seed weight. For the measurement of fresh weight of aerial parts, five to six week-old rice plants were grown on soil under various salt-stress conditions for 4 weeks. For the measurement of dry seed weight, three to four month-old rice plants were grown on soil under various salt-stress conditions for 2 months.Values are the means of ten replicates ±SD. The difference in the mean values between wild-type and ApDnaK rice plants was found to be statistically significant (* $P<0.01$).

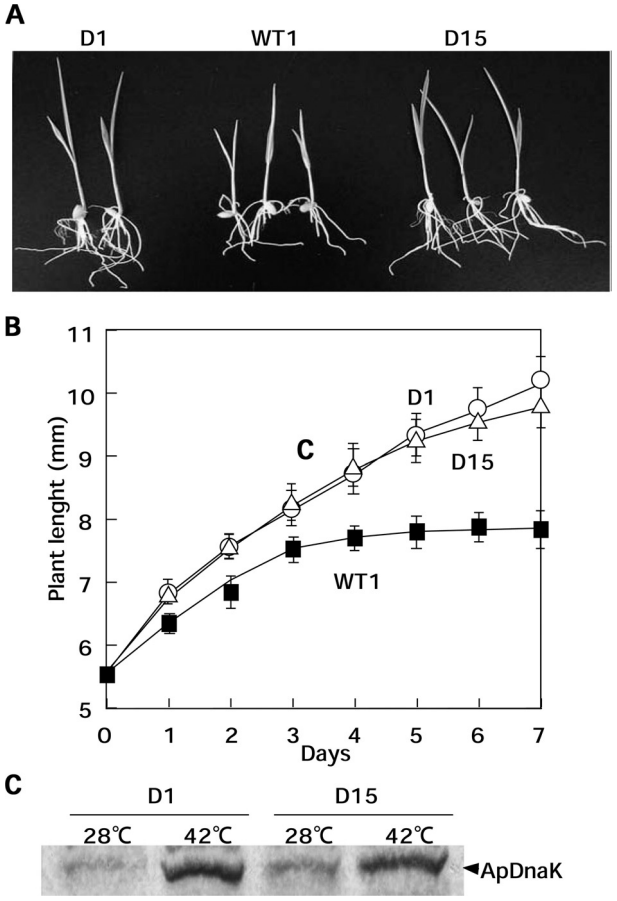

Figure 8. Effects of high temperature on the growth of the wild-type and transgenic rice plants. When the plant length reached 5.5 cm, the temperature of light period was increased to 42°C. Then plant length was measured. (A) Photographs of rice plants 7 days after high temperature treatment. (B) Effects of high temperature on the plant height. (C) Immuno blotting of ApDnaK before and after high temperature treatment. Values are the means of three replicates ±SD.

the wild-type plants (Figure 3). DnaK in *E. coil* has been shown to protect proteins from reactive oxygen species such as hydrogen peroxide (Echave et al. 2002). This has been interpreted as follows. Under abiotic conditions, *E. coil* proteins exist in a partially unraveled conformation and exposes their DnaK-binding domains. Therefore, ApDnaK would bind to proteins having partially unraveled conformation and change the conformation back into the properly folded form. In addition to the refolding activity by ApDnaK, binding of ApDnaK would protects the proteins from further attack of reactive oxygen species. It is remarkable that overexpression of ApDnaK increased the expression of APX and catalase that quench the active oxygen species, although it is not clear why overexpression of ApDnaK increased the expression of APX and catalase. Based on these functions, the overexpression of ApDnaK alleviated the decrease of seed yields in tobacco.

The most important finding in this study is the promotive effects of ApDnaK for the growth and seed yield in rice under our normal growth conditions (Figures 4–7). As shown in Figure 4D, the transgenic rice plants grew faster and bigger than wild-type plants. Western blot analysis showed the accumulation of ApDnaK proteins in the transgenic rice plants, but not in wild-type plants (Figure 4C). These results indicate that the overexpression of ApDnaK protein enhanced the size of rice plant. The results of Figure 5 shows that the overexpression of ApDnaK enhanced crop yield of rice plants. To clarify the reason for enhanced growth and seed yield in transgenic rice plants, we measured various parameters for photosynthesis. The results of Figure 6 showed that overexpression of ApDnaK enhanced the photosynthesis, activities of Calvin cycle, and sugar contents which would cause the growth enhancement under normal conditions. Similar results were also observed for the salt and high temperature stresses (Figures 7 and 8). To our knowledge, this is the first

example to show the growth promotion and increased seed yield as well as the stress tolerance by gene transfer technology.

Why did the seed yield increase under the normal growth conditions (14 h light at 28°C and 10 h dark at 23°C) in transgenic rice plants expressing halotolerant cyanobacterial DnaK? One possibility is that the above growth temperatures might be stressful for young rice seedlings. For instance, decrease of dry weight and chlorophyll contents grown at 28°C compared with those grown at lower temperatures have been reported (Shirasawa et al. 2006; Liu et al. 2007). Second possibility is due to the multi-functions of DnaK molecular chaperones. In addition to their function under stress conditions, HSPs can serve important functions on the processes of protein assembly, protein transport, and signal transduction under non-stress conditions (Hart 1996; Boston et al. 1996; Queitsch et al. 2000). Therefore, constitutive expression of highly active ApDnaK would facilitate the above described processes, thereby enhance the plant growth. Third possibility is that *A. halophytica* DnaK has high folding activity under nonstress conditions as well as the stress conditions when compared with the DnaK from *Synechococcus* sp. PCC 7942 (Hibino et al. 1999). Extra C-terminal amino acid sequence of ApDnaK has been shown to be involved in this activity.

It might be worthwhile to mention that the ApDnaK gene is not subjected to gene silencing because of low homology of amino acids between the DnaK from *A. halophytica* and those from plants (Lee et al. 1997a). It is plausible that the expression of ApDnaK did not affect the expression of rice Hsp70 as well as other Hsps belonging to different families in the transgenic plants (Lee et al. 1995). Therefore, the overexpression of ApDnaK in plants would cause the increase of maximum HSPs after heat shock.

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