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Technical Note

Ectopic expression of DnaK chaperone from a halotolerant cyanobacterium *Aphanothece halophytica* induced the bolting without cold treatment in *Eustoma grandiflorum*

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Abstract The DnaK/Hsp70 family is a molecular chaperone that binds non-native states of other proteins, and affects various physiological processes in bacterial, plant and animal cells. In this study, a *dnaK* gene from a halotolerant cyanobacterium, *Aphanothece halophytica*, was introduced into *Eustoma grandiflorum*. The *ApdnaK* transformed plants could grow at a similar rate with the control plants expressing the vector alone. Although exposure to low temperature is required for the bolting of control *Eustoma*, it was not required for the *ApdnaK* transformant. Under normal growth conditions, the glutathione content in the *ApdnaK* transformants was higher than that of control plants. In the transformant, the pistil is not evident and consequently, no seed formation. The results suggest the involvement of molecular chaperone for the bolting of *Eustoma*.

Key words: Abiotic stress, *Aphanothece halophytica*, bolting, DnaK, *Eustoma grandiflorum*.

Eustoma grandiflorum is a member of the family Gentianaceae and originated from Mexico and the southern regions of the USA (Roh and Lawson 1988). It is a relatively new floral crop in the ornamental industry. Many *Eustoma* cultivars with variations in flower color, size, and shape have been developed (Ichimura and Korenaga 1998), and is fast becoming a very popular cut flower in Japan.

One of the serious problems of *Eustoma* is the non-occurrence of rosette formation when plants were grown at a relatively high temperature (Ohkawa et al. 1994; Mino et al. 2003). Vernalization is required for the transition of *Eustoma* from vegetative rosette stage to the reproductive bolting stage (Ohkawa et al. 1994). Therefore, development of the species which bypass the rosette stage even at high temperatures has been highly desired. Recent studies have shown that a reduced glutathione (GSH) is a novel regulator of vernalization-induced bolting in *Eustoma grandiflorum* (Yanagida et al. 2004). GSH is an abundant and ubiquitous tripeptide in plant cells and plays an important role in the antioxidant system that detoxifies reactive oxygen species generated under stressful conditions including chilling stress (Yanagida et al. 2004).

Previous studies have shown that the overexpression of DnaK from a halotolerant cyanobacterium *Aphanothece halophytica* (ApDnaK) which was isolated from The Dead Sea, conferred the tolerance for salt (Sugino et al. 1999) and high temperature (Ono et al. 2001) in transgenic tobacco plants. It was also shown that the overexpression of ApDnaK in rice exhibited higher seed-yield compared with the wild-type plants under both normal and abiotic stress conditions (Uchida et al. 2008). DnaK protein is the prokaryotic analogue of eukaryotic heat shock protein 70 (Hsp70). The DnaK/Hsp70 is a molecular chaperone that binds non-native states of other proteins, and affects various physiological processes in bacterial, plant and animal cells. Therefore, this study aims to introduce an *ApdnaK* gene into *Eustoma grandiflorum* and examine its effect on the latter.

Seeds of *Eustoma grandiflorum* 'Suibijin' (Fukkaen-shubyo, Aichi, Japan) were sterilized with 2% sodium hypochlorite containing 0.02% TritonX-100 for 10 min and then germinated on the Murashige-Skoog (MS) agar medium. Plants were grown hydroponically in 10-fold-diluted MS medium in a growth chamber (Sanyo MLR-350HT, Japan) with a light (16 h at 200 μE m^{-2} s^{-1} at 22°C)/dark (8 h and 15°C) cycle with 70% humidity. *Eustoma* plants were transformed with the *ApdnaK* gene from *A. halophytica* by the *Agrobacterium*-mediated

Abbreviations: Ap, *Aphanothece halophytica*; CaMV, cauliflower mosaic virus; GSH, glutathione; Hsp, heat shock proteins; MS, Murashige and Skoog.
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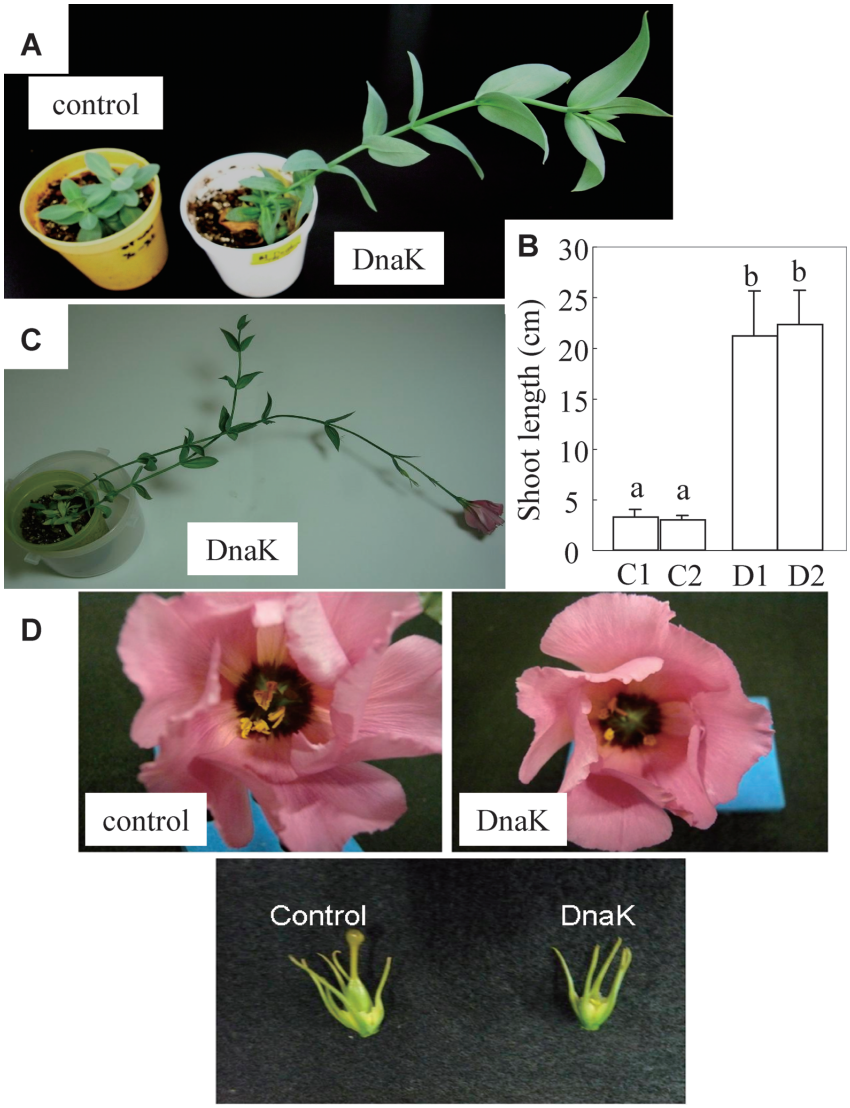

Figure 1. Morphogical and morpho-physiological changes of DnaK *Eustoma.* (A) Control and transformed plants/transformants grown at a constant temperature of 25°C for four months. (B) Shoot length after four months of control plants (C1, C2) and DnaK plants (D1, D2). Each value in Figure 1B shows the average of three independent measurements. Different letters denote significant differences at $p<0.05$ from control plants. (C) Transformant after 10 months grown at a constant temperature of 25°C. (D) Flower and ovary of control and transformants grown under normal light/dark cycle.

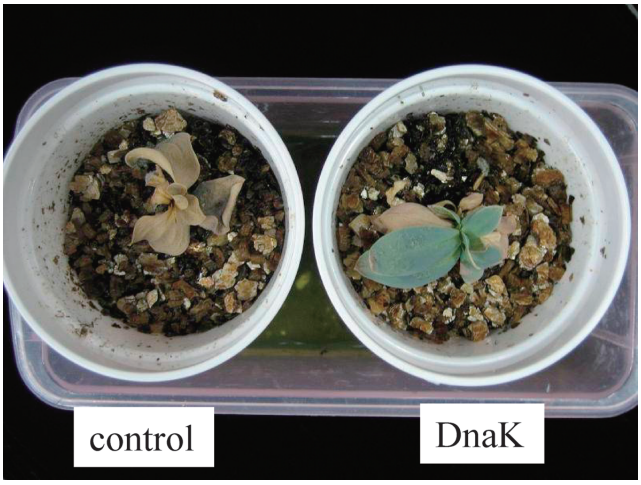

Figure 3. *Eustoma grandiflorum* after exposure to drought stress. Plants were grown for six weeks under normal light/dark cycle and then water was withheld for 30 days. After exposure to drought stress, plants were watered again for a week and then photographed .

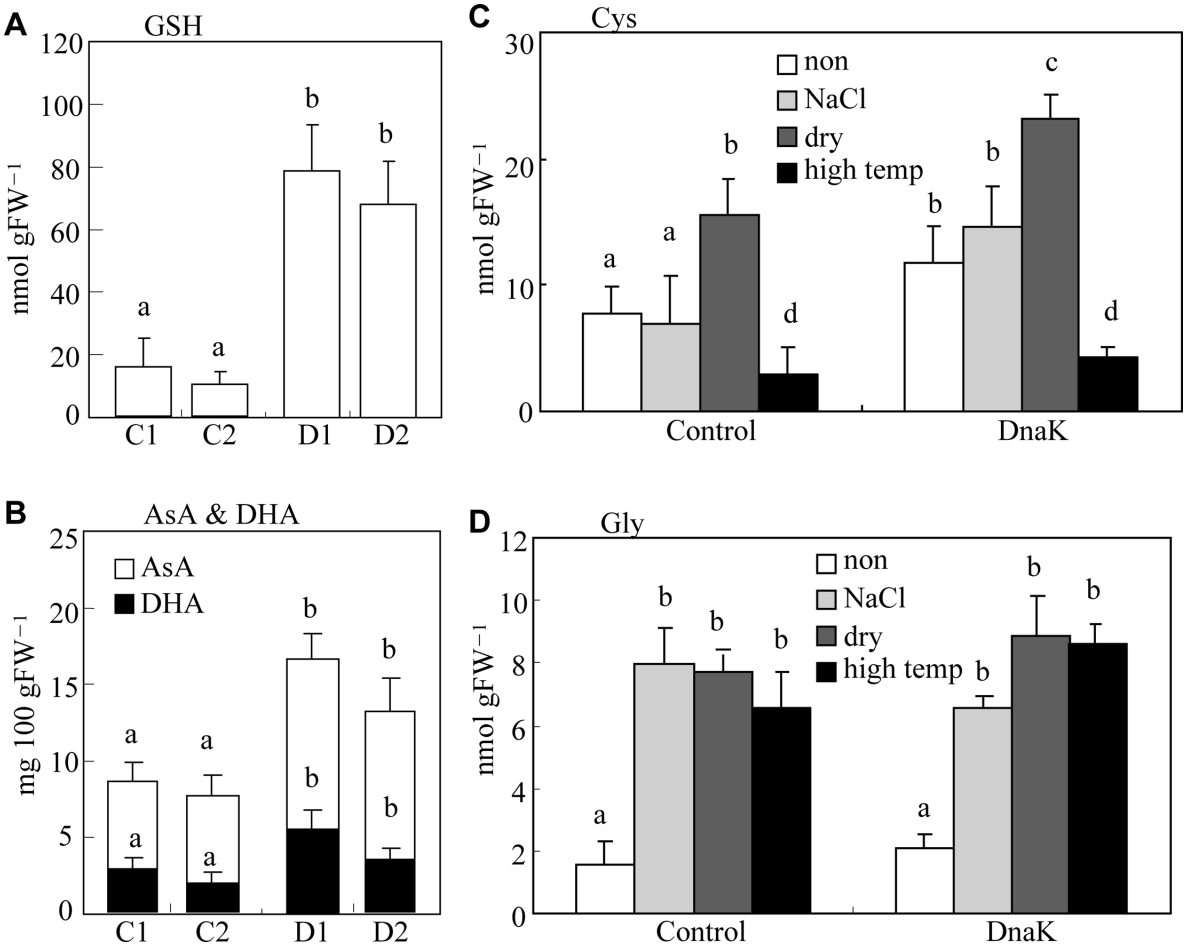

Figure 2. GSH, ascorbate-and amino acid contents. (A) GSH and (B) ascorbate (AsA and DHA) contents under normal condition. (C) Cys and (D) Gly contents under various stress conditions. GSH and ascorbate measurement were obtained from plants grown for four months at a constant temperature of 25°C while amino acid measurements such as Cys and Gly were obtained from plants grown for six weeks under normal light/dark cycle. Each value shows the average of three independent measurements. Different letters denote significant differences at $p<0.05$ from control plants. FW stands for fresh weight.

method as described by Uchida et al (2008). The binary vector pCAMBIA1301 containing the CaMV 35S promoter was used. Plants were grown aseptically on MS agar medium supplemented with 3% (w/v) sucrose containing 50 μg ml^{-1} kanamycin.

To measure GSH, plant leaves (1.0 g) were homogenized in 10 ml of 70% perchloric acid. The homogenate was filtrated and centrifuged at 15,000×g for 15 min at 4°C. The supernatant was adjusted to pH 7.0 with 3 M potassium phosphate and centrifuged at 15,000×g for 15 min at 4°C. GSH was measured using the DTNB (5,5′-dithiobis (2-nitrobenzoic acid)) termed as recycling method. The reaction mixture contained 10 mM sodium phosphate buffer, 0.25 mM NADPH, 0.75 units glutathione reductase and the extracted glutathione. The absorbance change at 412 nm was measured immediately after the addition of DTNB. GSH content was calculated from a standard curve obtained from authentic GSH. To measure the free amino acid residues, plants leaves (200 mg) were homogenized in 10 times of 90% methanol in a 1.5 ml Eppendorf tube. The homogenate was centrifuged at 15,000×g for 5 min at 4°C. The supernatant was dried up by centrifuge evaporation. Samples were dissolved, filtrated, and subjected under the Shimadzu Amino Acid Analysis System (Shimadzu Co., Kyoto, Japan).

Eustoma grandiflorum was transformed using the *Agrobacterium*-mediated method Gene integration and accumulation of ApDnaK were confirmed by PCR and Western blotting techniques, respectively (data not shown). Ten transgenic lines with vector alone (control plants) and with *ApdnaK* gene could be obtained, respectively. In contrast to the transgenic tobacco and rice, the transformed *Eustoma* with *ApdnaK* gene were not able to produce seeds. Two transgenic lines were maintained by producing the young seedlings from callus obtained from their respective mature leaves. Consequently, two transgenic lines were heterozygous. Southern blotting test showed they were independent (data not shown).

It is well known in *Eustoma* that vernalization is required for the transition from the vegetative rosette stage to the reproductive stage or bolting (Ohkawa et al. 1994; Yanagida et al. 2004). The study also confirmed this phenotype in the wild type and control *Eustoma* plants which were transformed with the empty vector. However, it was observed that bolting occurs in the transformants even without vernalization or low temperature treatment. Figure 1A shows the transformants which were grown at a constant temperature of 25°C. After four months, shoot length of the transformants about 22 cm whereas that of control plants about 3 cm (Figure 1B). Flowering was observed in transformants (Figure 1C) while the control *Eustoma* remained at rosette stage (data not shown).

When the plants were grown under normal light/dark cycle, flowering was observed in both control and transformed plants as shown in Figure 1D. However, seed formation was not evident in the transformants due to the absence of pistils (Figure 1D).

It has been reported by Oka et al (2001) and Yanagida et al (2004) that addition of GSH induced bolting even without vernalization. Therefore, the GSH content of the control and transformed plants which were grown four months at a constant temperature of 25°C was examined. As shown in Figure 2A, the level of GSH in the transformant was about 80 nmol gFW^{-1} whereas the GSH in control plants was about 18 nmol g^{-1} fresh weight (FW) which suggests that bolting in the absence of low temperature treatment in the transformants may be due to the increased level of GSH in the transformants.

Ascorbate is a major redox component in plants (Ishikawa et al. 2006). Therefore, the contents of ascorbate (AsA) and dehydroascorbate (DHA) (Yamamoto et al. 2005) was also examined. As shown in Figure 2B, the contents of both AsA and DHA are slightly higher in the transformants compared with that of control plants.

It has been shown that addition of Cys, a precursor of GSH, also induced bolting of *Eustoma* (Yanagida et al. 2004). As a follow up, the amino acid contents under various stress conditions was also measured. In this case, plants were grown six weeks under normal light/dark cycle, and then subjected to various stress. For drought-stress, six weeks old plants were grown without water for 20 days, then amino acid contents were measured. Salt stress was applied to plants by increasing the concentrations of NaCl stepwise 50 mM per every 3 days until it reached 200 mM, and then plants were allow to grow further for two weeks. For high temperature stress, plants were transferred to a growth chamber and exposed to 26°C temperature at for two weeks. The results of these experiments are shown in Figures 2C, D. As shown in Figure 2C, the Cys content in the transformants was higher than that of the wild type when plants were grown under control, salt-stressed, and drought-stressed conditions, but not under high temperature conditions. By contrast, the levels of Gly shown in Figure 2D and other amino acid residues (data not shown) in the transformants were almost the same to that of the control plants. These data indicate that the levels of Cys and GSH in the DnaK *Eustoma* were higher than that of control plants such that the transformants were induced to bolt even without exposure to vernalization treatment.

The stress tolerance of the DnaK *Eustoma* was also examined. For drought-stress, sixweek old plants were grown without watering. After 30 days, plants were watered again for a week to allow recovery. As shown in Figure 3, the control plant died whereas the DnaK *Eustoma* was able to survive. Similar results were also obtained for NaCl stress (data not shown).

In conclusion, the results indicate that ectopic expression of ApDnaK in *Eustoma* conferred the tolerance for drought and salt stresses. It was also observed that there is an increased level of GSH content and altered phenotype, specifically, bolting without vernalization in the transformants. The molecular mechanisms of these phenomena remain to be clarified.

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