J. Korean Grassl. Sci. Vol. 20(2) : 97~102, 2000

Increased Thermotolerance of Transgenic Rice Plant by Introduction of Thermotolerant Gene

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Abstract

To increase thermotolerance of forage crops, transgenic rice plants as a model for transformation of monocots were generated. A cDNA encoding the chloroplast-localized small heat shock protein (small HSP) of rice, Oshsp21, was introduced into rice plants via *Agrobacterium*-mediated gene transfer system. Calli induced from scutella were co-cultivated with a *A. tumefaciens* strain EHA101 carrying a plasmid, pIGhsp21. A large number of transgenic plants were regenerated on a medium containing hygromycin. Integration of Oshsp21 gene was confirmed by PCR and Southern blot analyses with genomic DNA. Northern blot and immunoblot analyses revealed that the Oshsp21 gene was constitutively expressed and accumulated as mature protein in transgenic plants. Effects of constitutive expression of the Oshsp21 on thermotolerance were first probed with the chlorophyll fluorescence. Results indicate that inactivation of electron transport reactions in photosystem II (PSII), were mitigated by constitutive expression of the Oshsp21. These results suggest that the chloroplast small HSP plays an important role in protecting photosynthetic machinery during heat stress.

(Key words : Thermotolerance, Rice, Transgenic, cDNA)

I. Introduction

Thermotolerance, or the ability of living organisms to withstand exposure to lethal high temperatures, is thought to be conferred by the induction of heat shock proteins (HSPs) (Lindquist and Craig, 1988). The major HSPs synthesized in eukaryotes are classified into five conserved classes, namely, HSP100, HSP90, HSP70, HSP60 and small HSPs of 15-30 kDa (Lindquist and Craig, 1988). Among these, the most abundant in higher plants is a group of small HSPs that belong to the HSP20 super family (Vierling, 1991; Waters, 1995).

Plant small HSPs are all nuclear-encoded and classified into five multigene families, being localized to different cellular compartments including the cytosol, the chloroplast, the endoplasmic reticulum (ER) and the mitochondrium (Waters et al., 1996). The chloroplast small HSP is synthesized in the cytoplasm as a precursor and transported into the chloroplast (Vierling et al., 1988). Little is known about the cellular function of these small HSPs. Recently, however, it has been shown that these proteins possess molecular chaperone activity to prevent thermal aggregation of proteins and to facilitate refolding of denatured proteins (Lee et al., 1995; Parsell and Lindquist, 1993; Waters et al., 1996). Several studies have demonstrated correlations between the accumulation of specific plant HSPs and thermotolerance (Chou et al., 1989; Lee et al., 1995; Park et al., 1996; Banzet et al., 1998). Thus, these observations suggested that small HSP may be

This work was supported by grant of Post-Doc. Program of Kyungpook National University, 1999.
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involved in the acquisition of plant thermotolerance.

Rice (*Oryza sativa* L.) is the most important cereal crop and rice straw has been also widely used as roughages in Korea. Rice is heat tolerant and is especially productive in summer. Most forage grasses cultivated in Korea are cool season grasses which have optimum temperature of 15~21℃. Among them, perennial ryegrass and timothy are known to be sensitive to high temperature. Temperatures above the optimum temperature greatly reduce growth of forages. For these reasons, it would be desirable to improve thermotolerance of heat sensitive forage grasses by introducing a thermotolerant gene.

To improve thermotolerance of forage grasses, transgenic rice plants that constitutively overexpress the chloroplast small HSP were generated. We also examined if overexpression of the small HSP would confer thermotolerance to transgenic plants.

II. Materials and Methods

1. Construction of Expression Vector

A cDNA encoding the chloroplast-localized small HSP, Oshsp21, was isolated from rice (*Oryza sativa* L. cv. Nagdongbyeo) (Lee et al., 2000). The *Xbal* (5') and *Xhol* (3') fragment of the Oshsp21 cDNA was placed downstream of the cauliflower mosaic virus (CaMV) 35S promoter within the pIG121-Hm vector (Hiei et al., 1994). The resulting plasmid, pIGhsp21, was introduced into *Agrobacterium tumefaciens*, EHA101 (Hood et al., 1986).

2. Plant Transformation

Dehusked mature rice seeds were sterilized in 2.5% NaOCl solution, inoculated into the callus induction medium (Toki, 1997), and incubated under light at 30℃. After 3 weeks, calli derived from the scutella were used for transformation. *Agrobacterium* containing the expression vector, pIGhsp21, was grown for 3 days on the AB medium containing 50 mg/L kanamycin and 50 mg/L hygromycin. The calli were soaked in this suspension for 2 min and excess bacteria were removed by blotting on sterile Kimwipes, and co-cultured for 3 days. After co-culture, the calli were washed with callus induction medium containing 50 mg/L carbenicillin and transferred to the selection medium containing 50 mg/L hygromycin and 50 mg/L kanamycin. The selected calli were transferred to the regeneration medium containing 1 mg/L NAA and 5 mg/L Kinetin (Toki, 1997).

3. PCR Analysis of Transgenic Plants

Genomic DNA was prepared from rice leaves by the cetyltrimethylammonium bromide (CTAB) method (Murray and Tompson, 1980). To confirm integration of expression vector, pIGhsp21, to transgenic plants, PCR technique was employed. For PCR analysis, genomic DNA from transgenic plant was used as a template and oligonucleotides 5'-CCCACCCACGAG-GAGCATC-3', corresponding to the sequence for the 3'-portion of the 35S CaMV promoter, and 5'-CACGCCGTTCTTGAGCTCG-3', corresponding to the internal sequence of Oshsp21 cDNA (Lee et al., 2000), were used as forward and reverse primers, respectively. The resulting PCR products were analyzed by electrophoresis on a 1.0% agarose gel.

4. Northern blot and Immunoblot Analyses

Total RNA was prepared from control or heat-treated leaves by the guanidine thiocyanate method (McGookin, 1984). Total RNA (5 μg) was subjected to electrophoresis on a 1% agarose gel containing 0.6 M formaldehyde, transferred onto a nylon membrane (Biodyne A, Pall BioSupport, NY) and hybridized with a ^{32}P-labeled Oshsp21 cDNA. Hybridization was performed at 42℃ for 16 h in a solution that contained 50% formamide. The membrane was washed with 0.2 × SSC and 0.1% SDS at 65℃ for 1 h, exposed to X-ray film or imaging plate and analyzed using an image analyzer

(BAS2000, Fuji Film, Tokyo).

Tissues were ground into a fine powder in liquid N_2 with a mortar and pestle. Soluble proteins were extracted and applied to 12% SDS-PAGE. After electrophoresis, gels were either stained with Coomassie brilliant blue R250 or transferred to nitrocellulose membrane (Protran, Schleicher & Schuell, Germany) for immunoblot analysis. Rabbit antiserum against a Oshsp21 fusion protein was used at a dilution of 1:2000 (Lee et al., 2000). Bound antibodies were detected using goat anti-rabbit alkaline phosphatase system (Promega, Madison, WI).

5. Chlorophyll Fluorescence Measurements

Rice plants were grown in a growth chamber under the conditions of 16 h light (25℃) and 8 h dark (22℃). Leaves were cut into discs with a diameter of 1 cm and placed on a temperature-controlled aluminum block. Chlorophyll fluorescence was measured with a modulation fluorometer, PAM101 (Walz, Germany) equipped with PAM103 and KL1500, a light source for saturating pulses (Miyao-Tokutomi et al., 1998).

III. Results and Discussion

To generate transgenic rice plants, the binary vector pIGhsp21 was introduced into rice plants through *Agrobacterium*-mediated gene transfer system using calli derived from the scutella. Initial selection of transgenic calli was carried out on a selection medium (Toki, 1997) containing hygromycin (50 mg/L) and kanamycin (50 mg/L). After 2 weeks on this medium, vigorously growing compact calli were transferred to the regeneration medium. From 98 selected calli 27 hygromycin-resistant plants were recovered and transferred to soil (Fig. 1).

To provide evidence of integration of the Oshsp21 into the rice genome, genomic DNA was isolated from transgenic rice plants and subjected to

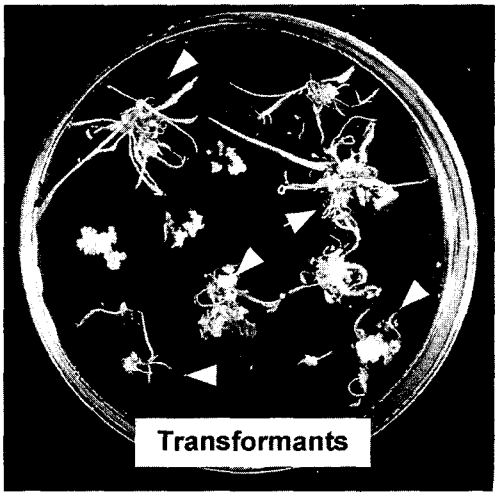

Fig. 1. Regeneration of transgenic rice plants. Photograph was taken 4 weeks after selected calli had been transferred to the regeneration medium.

Southern blot analysis. All transgenic plants analyzed gave the expected size of 1.2 kb band when genomic DNA was digested with *Xba*I (5') and *Sal*I (3') (data not shown). Subsequently, genomic DNA was prepared from 4 relatively vigorously growing transgenic plants and analyzed by PCR using specific primers targeting the junction of CaMV 35S promoter and Oshsp21. The expected 0.8 kb DNA fragment was amplified from genomic DNA isolated from all selected transgenic plants but not from non-transgenic plant (Fig. 2). These results indicate that the Oshsp21 gene in successfully integrated into genome of rice plants.

In order to examine the expression of the Oshsp21 gene in independent transgenic plants, total RNA was isolated from leaves of transgenic plants and subjected to Northern blot analysis (Fig. 3). Non-transgenic plant that had been treated at 42℃ for 2 h was used as a control. As shown in Fig. 3A, transcripts were not detected in non-transgenic plant at the control temperature of 25℃, but were accumulated at 42℃ for 2 h. By contrast, significant levels of the transcripts were accumulated in all

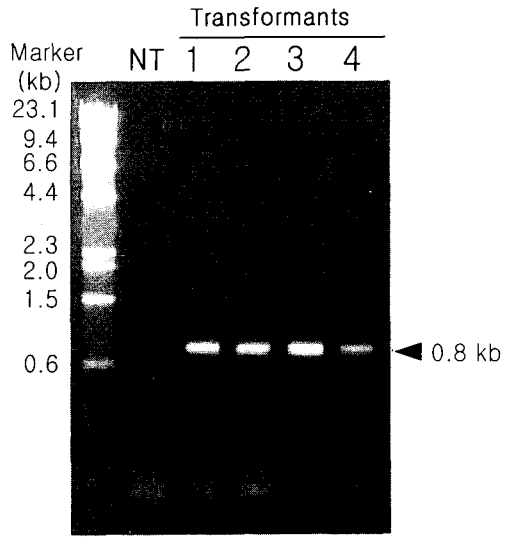

Fig. 2. PCR analysis with genomic DNA in transgenic plants. Genomic DNA was isolated from transgenic plant and used as a template. After PCR amplification, PCR products were analyzed by agarose gel electrophoresis. NT, non-transgenic plant; Tr, transgenic plant. Arrowhead indicates 0.8 kb DNA fragments amplified by PCR.

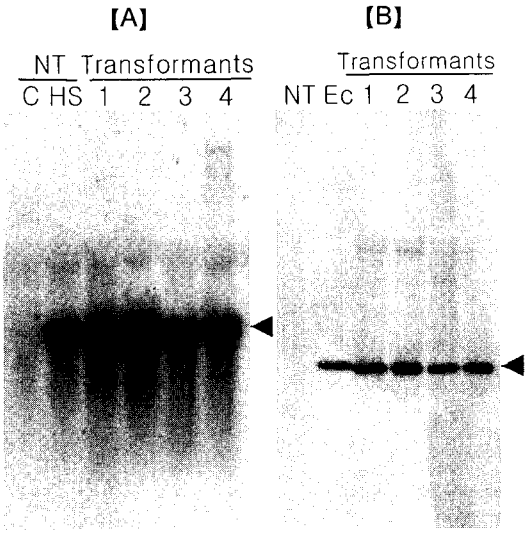

Fig. 3. Northern blot and immunoblot analyses of transgenic plants. [A] Northern blot. Total RNA (5 μg) was fractionated by agarose gel electrophoresis and subjected to northern hybridization. NT. non-transgenic plant. [B] Immunoblot. Soluble protein was purified from leaves and subjected to immunoblotting.

transgenic plants at control temperature. Three out of 4 transgenic plants (transformants 1, 2 and 4) accumulate considerably more Oshsp21 transcript in the absence of heat stress than do heat-stressed (42 ℃, 2 h) non-transgenic plants.

The accumulation of OsHSP21 protein in transgenic plants was analyzed by immunoblotting using anti-OsHSP21 protein antibody (Fig. 3B). The antibody detected a single band of 21 kDa in cell lysates of *E. coli* that expressed the mature OsHSP21 protein. Also in heat-treated leaves of non-transgenic plants, a single band of 21 kDa was detected. These observation indicates that the precursor OsHSP21 protein expressed on heat stress was processed to the mature form in leaves. This 21 kDa band was also detected in leaves of all transgenic plants. As in the case of the transcript level, the levels of OsHSP21 protein were different among transgenic plants. These results indicate that the Oshsp21 gene was constitutively expressed and normally accumulated as mature protein in transgenic plants.

To determine effects of constitutive expression of the OsHSP21 protein on thermotolerance were first probed with the chlorophyll fluorescence. After 5-min incubation of leaf discs at high temperatures, the Fo, Fv and Fm levels were determined and the Fv/Fm ratio that has been used as parameter of the physiological state of photosynthetic apparatus in intact leaves was calculated (Pospisil et al., 1998). As shown in Fig. 4, decrease in Fv/Fm at high temperature was mitigated by constitutive expression of the Oshsp21. In non-transgenic plant, Fv/Fm at 50℃ was decreased to 10% of control temperature of 25℃. By contrast, Fv/Fm at 50℃ in transgenic plant remained about half of control temperature.

These observations indicated that thermotolerance of

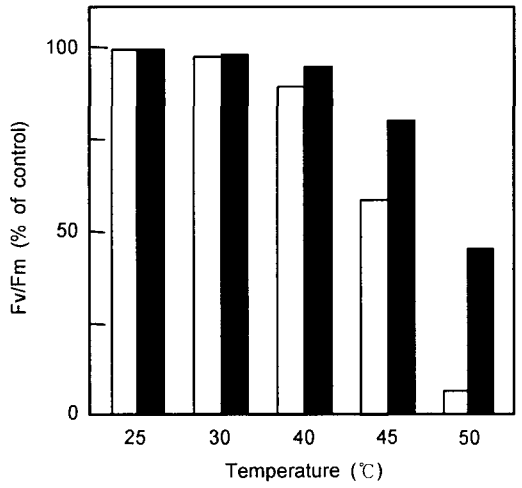

Fig. 4. Changes of photosynthetic parameter, Fv/Fm. Chlorophyll fluorescence after 5-min heat treatment at designated temperatures. Percent increase of Fv/Fm was represented.

photosystem II (PSII) was improved by the constitutive expression of the Oshsp21.

Results in this study suggest a possibility that establishment of gene transfer system for thermotolerance genes into monocots could be a useful tools to improve thermotolerance of heat sensitive forage grasses.

IV. References

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