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Acquisition of Thermotolerance in Transgenic Orchardgrass Plants with *DgHSP17.2* Gene

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ABSTRACT : To develop transgenic orchardgrass (*Dactylis glomerata* L.) resistant to high temperature, the recombinant *DgHSP17.2* gene was introduced into orchardgrass plants using the *Agrobacterium*-mediated transformation method and expressed constitutively under the control of the CaMV 35S promoter. The results of genomic DNA PCR and Southern analysis showed a DNA band and hybridization signal on agarose gel and X-ray film in transgenic orchardgrass plants harboring the recombinant *DgHSP17.2* gene, but a DNA band and hybridization signal were not observed in the wild type and empty vector control plants. The same result was also obtained in RT-PCR and Southern blot analysis, and these transgenic orchardgrass plants did not show any morphological aberration both in the culture bottle and soil mixture. When leaf discs cut from transgenic orchardgrass plants with recombinant *DgHsp17.2* gene were exposed to lethal temperature (heat treatment at 60°C for 50 min), 60-80% of the leaf discs showed only damage symptoms, but non-transgenic leaf discs showed a lethal condition. These results indicate that the *DgHsp17.2* gene may act as a protector from heat stress in plants. (**Key Words** : Orchardgrass (*Dactylis glomerata* L.), *Agrobacterium*-mediated Transformation, *DgHSP17.2* Gene, Thermotolerance)

INTRODUCTION

Orchardgrass (*Dactylis glomerata* L.) is one of the most important perennial grasses grown in Korea. It is quite vigorous in growth and rapid in establishment and recovery after cutting or grazing, but susceptible to heat stress in Korea. We conducted this study to develop high tolerant orchardgrass plants. Since exposure to high temperature represents a serious threat to cellular viability, organisms have developed a response mechanism by synthesizing a group of proteins among which are mostly molecular chaperones, assisting other cellular proteins to function correctly. Small heat-shock proteins (sHSPs) represent an abundant and ubiquitous family of molecular chaperones. In contrast to other chaperone families (e.g., Hsp100, Hsp90, Hsp70, and Hsp60), the sHSP family is characterized by having a conserved "α-crystalline domain" and a low molecular mass per subunit (12-42 kDa) (de Jong et al., 993; Fink, 1999; Narberhaus, 2002; van Montfort et al., 2002).

The protein aggregates are toxic to the cells since they impair normal cellular functions (Horwich, 2002). According to the current proposed model, sHSPs prevent the irreversible protein aggregation and insolubilization under stress conditions by binding these non-native proteins to form a soluble complex (Horwitz, 1992; Jakob et al., 1993; Chang et al., 1996; Haslbeck et al., 1999; Lee and Vierling, 2000). The *in vitro* chaperone activities of sHSPs are therefore usually determined by their capacity to suppress thermally or chemically induced aggregation of some model proteins (Horwitz, 1992; Jakob et al., 1993). Enormous efforts have been made to elucidate the associated mechanisms of sHSPs (Feder and Hofmann, 1999; Narberhaus, 2002; van Montfort et al., 2002; Thomas et al., 2005).

In a previous study (Kim et al., 2002), the *BcHSP17.6* cDNA for a low molecular weight heat shock protein (LMW HSP) was isolated from Chinese cabbage (*Brassica campestris* M.). When soluble proteins isolated from *E. coli* transformed with *BcHSP17.6* cDNA were heated at 55°C for 30 min, the *BcHSP17.6* protein was shown to stabilize against heat denaturation *in vitro*. In the present study, in order to develop orchardgrass tolerant to summer

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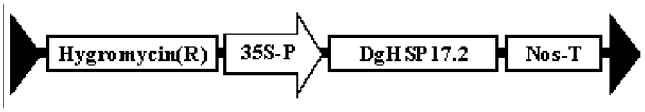

Figure 1. Construction of overexpression vector, Pcambia 1300PT/DgHSP17.2, for plant transformation. 35S-P, CaMV 35S promoter; DgHSP17.2, *DgHSP17.2* gene (GenBank accession number DQ172835); Nos-T, Nos terminator.

depression, we attempted to produce transgenic orchardgrass plants harboring expressed *DgHSP17.2* gene constitutively using the *Agrobacterium*-mediated transformation method. We have examined whether the transcripts of *DgHSP17.2* gene are expressed in transgenic orchardgrass plants.

MATERIALS AND METHODS

Plant materials, bacteria strains and culture medium

Callus was initiated from mature seeds of orchardgrass (*Dactylis glomerata* L., cv. Potomac). Binary vector pCAMBIA1300PT under the control of the CaMV (cauliflower mosaic virus) 35S promoter was used for constructing pCAMBIA/DgHSP17.2. This construct was mobilized into *Agrobacterium tumefaciens* strain GV3101. *Agrobacterium* was grown on YEP (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, pH 7.2) medium at 28°C for approximately 2 days.

Vector (pCAMBIA1300PT/DgHSP17.2) for transformation of orchardgrass plants

For vector construction of pCAMBIA1300PT/DgHSP17.2, for orchardgrass transformation, about 0.9 kb *DgHSP17.2* sequence was ligated into pCAMBIA1300PT under the CaMV 35S promoter (Figure 1). The *DgHSP17.2* gene was isolated from orchardgrass (GenBank accession number DQ172835).

Regeneration of transgenic orchardgrass plants

For regeneration of transgenic orchardgrass plants, vector constructs were transferred into *Agrobacterium* by the freeze-thaw method (Holster et al., 1978). Transformation and regeneration of ochardgrass and tall fescue was performed by procedures described in Lee et al. (2001), Kim et al. (1998b), and Lee et al. (2004). *Agrobacterium*-infected orchardgrass callus were placed on ONCoC solid medium (N6 salt, vitamin mix, 3% sucrose, 1% glucose, 100 μM acetosyringone, 0.2% gelrite, pH 5.2), and then co-cultured at 28°C in the dark for three days. After that, these were transferred to a callus selection medium (ONCS: N6 salt, vitamin mix, 3% sucrose, 2% casein, 2 mg/L dicamba, 0.5% gelrite, 250 mg/L cefotaxime, 30 mg/L hygromycin, pH 5.8). After about five weeks, calli were transferred to regeneration medium (ONPR: N6 salt, vitamin mix, 2% sucrose, 2% sorbitol, 2% maltose, 1 mg/L NAA, 5 mg/L kinetin, 2.5 mg/L dopper, 0.5% gelrite, 250 mg/L cefotaxime, 50 mg/L hygromycin, pH 5.8). For root development and regeneration of transgenic orchardgrass plants, induced shoots were grown in N6 medium containing a half of N6 salt. Regenerated transgenic plants were transferred to soil mixture for further analysis.

Genomic DNA PCR analysis

Genomic DNA was extracted from orchardgrass plants using the CTAB method (Murray and Tompson, 1980). For PCR identification of transgenic orchardgrass plants, the primer set utilized was 35S-s1 (5'-TTCAACAAAGGGTAATATCCGG-3') as a forward primer and DgHSP-s2 (5'-GCGTCGACTCACTCACTAA TCATCGA-3') as a backward primer. PCR amplification was performed in a Personal Cycler (Biometra, Germany) with ExTaq polymerase (Takara, Japan). PCR reaction was performed using the following cycling parameters; 1 cycle of 1 min at 95°C, 35 cycles of 1 min at 95°C, annealing of 1 min at 55°C and final extension of 1 min at 72°C.

RNA preparation and reverse-transcription (RT)-PCR

To test the expression of *DgHSP17.2* transcript in transgenic orchardgrass plants, total RNA was extracted using Guanidine Thiocyanate method (McGookin, 1984). RT reaction was performed for 1 h at 42°C with 2 μg of total RNA using DgHSP-s2 (5'-GCGTCGACTCACTCAC TAATCATCGA-3') primer. Immediately PCR reaction was performed using the following cycling parameters; 1 cycle of 2 min at 95°C, 35 cycles of 1 min at 95°C, 1 min at 55°C, 2 min at 72°C and final extension of 10 min at 72°C. The primer sets used for PCR were primers DgHSP-s1 (5'-GCGGATCCATGGAGGGCAGGGTGTTC-3') and DgHSP-s2 combination. Amplified RT-PCR products were electrophoretically separated on 1.2% agarose gels, and were visualized by ethidium bromide (EtBr) staining.

Southern blot analysis

Southern blot analysis was achieved using PCR and RT-PCR products. Separated DNA on 1% agarose gel was transferred onto positively charged nylon membrane (Amersham pharmacia biotech). After the transfer to nitrocellulose membranes, filters were prehybridized at 42°C for 1-2 h in 50% formamide, 5× SSPE, 5× Denhardt's solution, 0.1% SDS, and 0.1 mg/ml denatured salmon sperm DNA. The hybridization to the probe labeled with [α-^{32}P] dCTP was done overnight in prehybridization buffer. Filters were washed twice at room temperature for 10 min in 2× SSC and 0.1% SDS, once at 65°C for 15 min in 1× SSC and 0.1% SDS, and twice at 65°C for 15 min in 0.1×

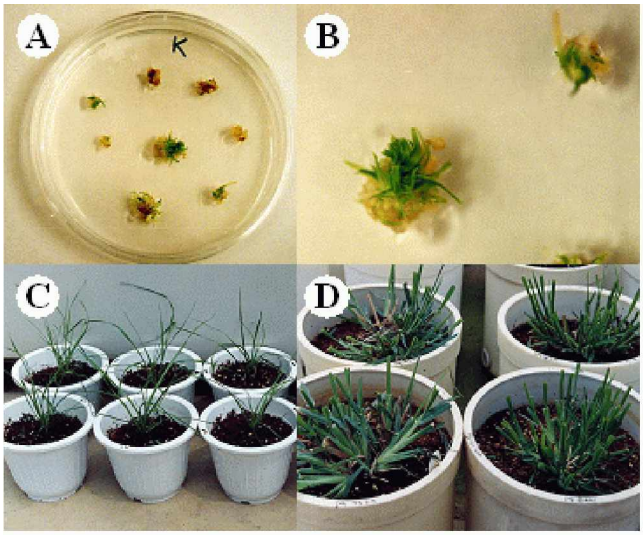

Figure 2. Plant regeneration from transgenic callus on regeneration medium containing 50 mg/L of hygromycin and acclimation of transgenic plants in greenhouse. A and B, Regeneration step; C and D, Transgenic plants.

SSC and 0.1% SDS. Probe for the analysis was prepared from the *DgHSP17.2* DNA fragment, and then labeled with [α-^{32}P] dCTP.

Thermo-tolerance test of transgenic orchardgrass plants

To determine the lethal temperature of orchardgrass (*Dactylis glomerata* L. cv. Potomac), leaf discs cut from non-transgenic 4 weeks-old orchardgrass seedlings were treated at 45, 50, 55 and 60°C for 60 min. Then new leaf discs were treated at 60°C for 30, 40 and 50 min. According to the determined lethal temperature, leaf discs cut from transgenic and non-transgenic orchardgrass plants were exposed to lethal temperature.

Nucleotide sequence accession number

The sequence data for the *DgHSP17.2* gene has been assigned GenBank accession number DQ172835.

Abbreviations

DgHSP17.2, a thermotolerance gene isolated from *Dactylis glomerata*; sHSP, small heat shock protein; CTAB, cetyl trimethyl ammonium bromide; PCR, polymerase chain reaction; RT-PCR, reverse-transcription PCR.

RESULTS AND DISCUSSION

Production and confirmation of transgenic orchardgrass plants

The plasmid pCAMBIA1300PT/DgHSP17.2 was constructed by subcloning a *DgHSP17.2* fragment into pCAMBIA1300PT plasmid. *A. tumefaciens* GV3101 was transformed with constructed pCAMBIA1300PT/

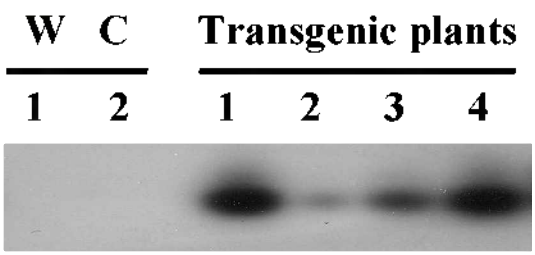

Figure 3. Confirmation of transgenic plants by PCR and Southern blot analysis using genomic DNAs from wild-type (W), transgenic empty vector coltrol (C) and transgenic plants with recombinant *DgHsp17.2* gene (four lines of DgHSP17.2).

DgHSP17.2. The transformed colonies were selected on YEP medium containing 50 μg/ml of hygromycin and 100 μg/ml of rifampicin. This recombinant plasmid pCAMBIA1300PT/DgHSP17.2 was used for production of transgenic orchardgrass plants with expression of *DgHSP17.2* cDNA driven by 35S promoter.

Transgenic plants were regenerated from hygromycin-selected calli on regeneration medium containing 50 mg/L of hygromycin and were acclimated in a greenhouse (Figure 2). Transgenic plants did not show any morphological difference from wild-type plants. To confirm the integration of the *DgHSP17.2* gene into the orchardgrass genome, transgenic plants were analyzed by genomic DNA PCR and Southern blot analysis.

Genomic DNA PCR of wild type control and transgenic orchardgrass plants was performed using 35S-s1 (5'-TTCAACAAAGGGTAATATCCGG-3') and DgHSP-s2 (5'-GCGTCGACTCACTCACTAATCATCGA-3'), and Southern analysis was performed using *DgHSP17.2* DNA fragment as a probe. The result of Southern blot analysis showed obvious hybridization signals on X-ray film, but no hybridization signals were observed in the wild type and empty vector control plants (Figure 3). These Southern analysis results confirmed insertion of recombinant *DgHSP17.2* gene into the orchardgrass genome. Forty two generations of transgenic lines harboring the recombinant *DgHSP17.2* gene were selected for further analysis. These transgenic plants were rooted in culture bottles and then were transferred to soil mixture for RNA analysis and thermo-tolerance testing.

RT-PCR and Southern blot analysis

Expression of recombinant *DgHSP17.2* gene in transgenic orchardgrass plants was detected by RT-PCR and Southern blot analysis (Figure 4). No transgene expression was observed in wild type plants. Among 40 plants in which transgene insertion into the plant genome was confirmed, five plants showed no or weak transgene expression. In spite of the transgene expression level, transgenic orchardgrass plants did not showed any morphological

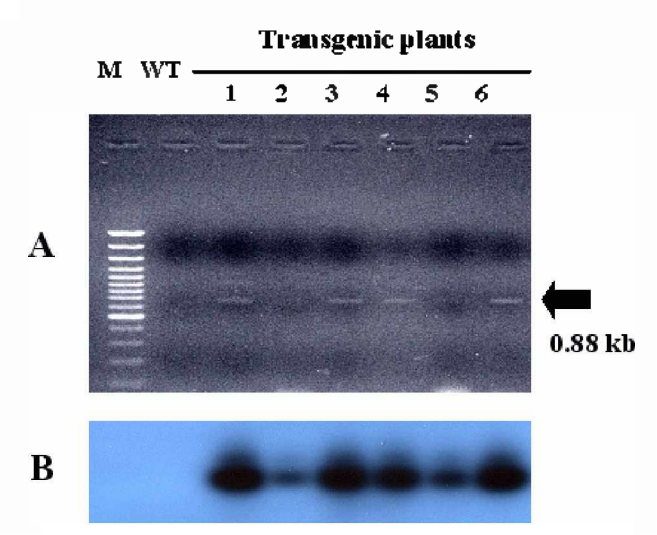

Figure 4. Confirmation of transgenic plants by RT-PCR and Southern blot analysis using genomic DNAs from wild-type (WT) and transgenic plants. The numbers (1-6) indicate independent transgenic lines. (A) Agarose gel electrophoresis using RT-PCR products. (B) Southern blot analysis using RT-PCR products.

aberration both in the culture bottle and soil mixture.

Thermotolerance test of transgenic orchardgrass plants

When leaf discs cut from non-transgenic orchardgrass were treated at 45, 50 and 55°C for 60 min, they showed different damage symptoms but the conditions were not lethal. By contrast, most leaf discs showed lethal conditions within one day after heat treatment at 60°C for 60 min. Therefore, new leaf discs were treated at 60°C for 30, 40 and 50 min, respectively. After three days following heat treatment at 60°C for 40 and 50 min, leaf discs showed lethal conditions at 60°C for 50 min, but showed no lethal condition at 60°C for 30 and 40 min. These results indicated that heat treatment at 60°C for 50 min was an optimum condition to distinguish the lethality of orchardgrass leaf discs (Table 1). In Table 1, ++++ means leaf discs appeared as healthy as unheated control, +++ means almost leaf discs

Table 1. Determination of heat-lethal temperature in leaf disc of orchardgrass plant (*Dactylis glomerata* L.)

Days after heat treatment	Time of heat treatment at 60°C		
	30 min	40 min	50 min
1	++++	+++	+
2	+++	++	-
3	++	++	-

Leaves of plant from several experiments were scored on day 3.

appeared as healthy as unheated control with some yellow tissue, ++ means most leaf discs had some bleached and withered tissue, + means most leaf discs brownish and green tissue was still evident after 3 days, and -means all leaf discs brownish within 3 days.

When the leaf discs cut from transgenic orchardgrass plants with recombinant *DgHsp17.2* gene were exposed to lethal temperature, that is, heat treatment at 60°C for 50 min, 60-80% of the leaf discs showed only damage symptoms, but non-transgenic leaf discs showed a lethal condition (Figure 5) as expected. This result suggest that the *DgHsp17.2* gene introduced to orchardgrass plants is related to thermotolerance, and that the 17.2-kD HSP acts as a protector from heat damage in plants, that is, the 17.2-kD HSP may act as a 'molecular chaperone'. If this hypothesis is correct, cereal species, vegetables and other cultivated plants which are sensitive to high temperature will be better cultivated and produced under high temperature conditions by transformation with this *DgHsp17.2* gene.

Lin et al. (1984) suggested that the sHSPs were required for thermoprotection of soluble proteins. Jinn et al. (1995) suggested that the sHSPs provided a very significant thermostabilization of soluble proteins against heat denaturation at 55°C for 30 min, and the degree of protection was proportional to the amount of this protein added. In our previous study (Kim et al., 1997; Kim et al., 1998a; Kim et al., 2002), *BcHSP17.6* cDNA was introduced into tobacco plants and 17.6-kd HSP was detected in transgenic tobacco plants. In particular, transgenic tobacco plants were slightly damaged or not damaged at heat-killing

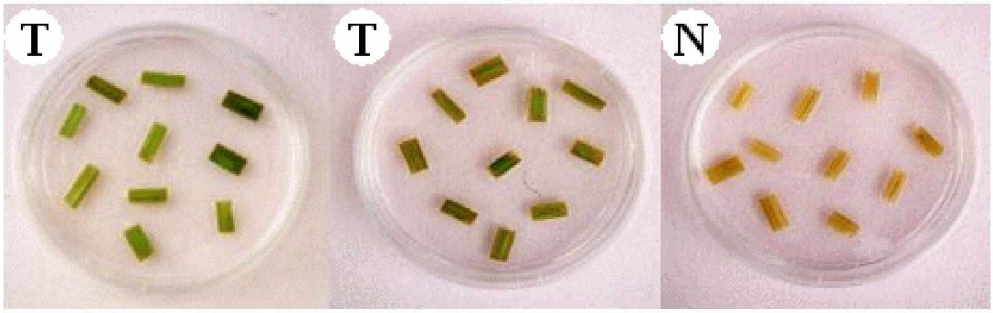

Figure 5. Thermo-tolerance test of transgenic orchardgrass plants. When the leaf discs cut from transgenic plants with recombinant *DgHsp17.2* gene (T) or non-transgenic (N) orchardgrass (*Dactylis glomerata* L.) plants (N) were exposed to lethal temperature by heat treatment at 60°C for 50 min, 60-80% of the transgenic leaf discs showed only damage symptoms, but non-transgenic leaf discs showed lethal conditions.

temperature (exposure at 50°C for 15 min.). This result agreed well with the above results and it was concluded that accumulation of the sHSPs is necessary for plants to survive at an otherwise lethal high temperature.

Recently, sHSPs were demonstrated to form a network together with Hsp70 and Hsp100 to efficiently refold the non-native proteins from the protein aggregates (Mogk et al., 2003a, b; Cashikar et al., 2005; Haslbeck et al., 2005). Hsp70 alone was shown to be able to mediate the refolding of substrates from the soluble complex, whereas Hsp100 was essential for the refolding of substrates from the insoluble complex (Haslbeck et al., 2005). Meanwhile, it was found that Hsp100 was critical for the thermal tolerance of yeast or bacterial cells (Lindquist and Kim, 1996; Thomas and Baneyx, 1998; Weibezahn et al., 2004). This suggests that, upon the heat-shock stress, the insoluble protein aggregates indeed exist and the elimination of such protein aggregates by the multi-chaperone network is critical for the viability of cells. The presence of sHSPs in protein aggregates may represent the evolutionary income of organisms to efficiently remove such toxic factors.

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