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# HETEROLOGOUS EXPRESSION OF MSHSP23, A *MEDICAGO SATIVA* SMALL HEAT SHOCK PROTEIN, ENHANCES HEAT STRESS TOLERANCE IN CREEPING BENTGRASS

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## ABSTRACT

Small heat shock proteins (Hsps) are conserved in living organisms. They exist in diverse subcellular organelles and play important roles in plant defense systems against various abiotic stresses. Chaperone properties of small Hsps have been widely known to prevent the stress-induced denaturation of substrate proteins in cells. Here, we examined the capacity of*MsHsp23*to confer tolerance to heat stress (thermotolerance)by heterologous expression in creeping bentgrass. We generated nine independent transgenic creeping bentgrass plants. Two independent transgenic plants (Tg-1 and Tg-2) were examined forthermotolerance (42°C/24h).This resulted in minimal wilting ofthe leaves, which retained healthy green color, while the non-transformed (NT) plants wilted and showed light-green color of their leaves. Plants overexpressing *MsHsp23* displayed higher ascorbate peroxidase (APX) activities. Therefore, we conclude that heterologous expression of *MsHsp23* in creeping bentgrass can protect plants against heat stress, presumably bychaperone activity that allows for induction of APX.

**Keywords:** Small Heat-shock protein, Thermotolerance, Creeping bentgrass, Antioxidant enzymes.

## INTRODUCTION

Heat-shock, caused by un-physiologically high temperature, induces expression of genes encoding heat-shock proteins (Hsps) in prokaryotic and eukaryotic organisms (Vierling, 1991). Among these proteins, small Hsps with a molecular mass of 12 to 43 kDa are classified into 6 major multigene families based on their different subcellular localization, with two sub-families located in the cytoplasm, and one each in chloroplasts, mitochondria, the endoplasmic reticulum and the plasma membrane (Iba, 2002; Wang *et al*., 2004). A distinct structural feature of small Hsps is a conserved centrally located α-crystallin domain.. However, N-terminal and C-terminal regions are not well conserved, are of variable in length and show divergent sequences (Narberhaus, 2002; Reddy *et al*., 2006). Small Hsps have been known to associate into homo-multimeric complexes ranging from 200 to600 kDa in plants and mammals (Narberhaus, 2002; Sun *et al*, 2002). Usually, small Hsps in plants not only form 12 subunits of homo-oligomers, but also form hetero-oligomers with different classes of small Hsps*in vitro* and *in vivo* (Helm *et al*., 1997; Kirschner*et al*., 2000). Such high molecular weight oligomeric structure formations are enhanced by elevated temperatures (Kirschner*et al*., 2000). The distinct functional characteristics of small Hsps function as molecular chaperones that can prevent irreversible protein aggregation and lack of solubility. Stress-induced high oligomeric complexes of small Hsps show enhanced substrate binding capacity that prevents substrate aggregation, thereby supporting stress resistance to cells and protection the entire organism (Sun *et al*., 2002; Wang *et al*., 2004; Siddique *et al*., 2008).

In plants, it has been known that various stress conditions, such as heat, chilling,drought, toxic metals and oxidative injury, cause the induction of small Hsps(Malik *et al*., 1999; Lee *et al*., 2000; Hamilton and Heckathorn, 2001; Lopez-Matas*et al*., 2004; Sato and Yokoya, 2008).We previously have identified two small Hsps, representing rice chloroplast-localized small Hsp26 (*OsHsp26*) and alfalfa mitochondrial Hsp23 (*MsHsp23*).When *OsHsp26*was overexpressed in tall fescue plants,it achieved tolerance to oxidative and heat stress.Overexpression of *MsHsp23* confers tolerance to salinity and arsenic stresses in tobacco and tall fescue plants with enhanced antioxidant enzyme activities (Kim *et al*., 2012; Lee *et al*., 2012a,b).

Most perennial crops have been adapted to cool-seasons growth with optimum growth temperature around 21° C. Thus, they are susceptible to heat stress and survival and productivity in particular are dramatically depressed duringthe warmseason, referred as a summer-depression (Seo*et al*., 1988). Plant sensitivity during the summer depression period of perennial crops is also tightly related to winter hardening (Van Santen and Sleper, 1996). Here we report on the construction of transgenic creeping bentgrass plants overexpressing *MsHsp23 t*hatshow increased heat stress tolerance, which

may be related to the chaperone activity of small heat-shock proteins.

## MATERIALS AND METHODS

**Plant materials and growth conditions:**Mature seeds of creeping bentgrass (*Agrostis stolonifera* L.), cultivar Penncross, were used. The seeds were de-husked manually, rinsed with 70% ethanol for 1-2 min, and washed with distilled water three times.And then, the seeds were then surface-sterilized by immersion for 30 minutes in a solution of 30% (w/v) sodium hypochlorite and 0.1% of Tween-20. To remove the surfactants, sterilized seeds were rinsed ten times with sterile deionized-distilled water. Callus induction and *Agrobacterium*-mediated genetic transformation were performed according to our previous protocol (Lee *et al*., 2011).

**Vector construction and genetic transformation:**The alfalfa (*Medicago sativa* L.) small Hsp23 (*MsHsp23*)cDNA fragment was ligated into the translation initiation codon within the *5′* untranslated sequence of the constitutively expressed cauliflower mosaic virus promoter (35S). The resultant chimeric gene cassette was then inserted into the *Kpn*I/*Xba*I site of a pCAMBIA1300 binary vector (Fig. 1). Recombinant pCAM-*MsHsp23* was introduced into *Agrobacterium tumefaciens*strain EHA105, which was used for genetic transformation (Lee *et al.*, 2012a, b).

**Selection and Molecular analysis of transgenic plants:**Genomic DNA was isolated from the leaves of wild-type and transgenic creeping bentgrass plants using CTAB. Putative hygromycin-resistant transgenic plants were selected by PCR analysis. To verify the presence and integration of the *MsHsp23* and *HPT*genes, all selected plantlets were subjected to molecular analyses by PCR and Southern blotting.

PCR was performed using the following primers: 5'-TCTAGAATGGCGTCTGTTGCT-3' and 5'-TTCTCAGCTATTTAGGAGCTC-3 for the *MsHsp23* gene while primers '; 5'-CCTGAACTCACGACG-3' and 5'-AAGACCAAGGAGCATAT-3' for the *HPT*gene. PCR amplification was carried out according to our previous protocol (Lee *et al*., 2012a).

Integration of the *MsHsp23* gene into the creeping bentgrass plants genome was further confirmed by Southern blotting analysis. Twenty micrograms of genomic DNA was digested with *Eco*RI, separated electrophoreticallyon 1.0% agarose gel, transferred to a nylon membrane and hybridized with the [α-32P]-dCTP-labeled *MsHsp23* probe by PCR-based labelling using pCAM-*MsHsp23* plasmids as a template. Southern blot hybridization and membrane washing were performed as previously described (Lee *et al.*, 2000).

**Thermotolerance Test:**To examine whether the overexpression of the *MsHsp23* gene would displayed enhancedthermotolerance in the transgenic creeping bentgrass plants, three independent lines of transgenic plants and wild type plants were transplanted into soil pots in growth chamber (25°C)and subjected to the thermotoleranceassay. Heat stress was applied by raising the temperatures to 42°C for 24h in the growth chamber. Following heat treatment, the plants were maintained for 7days under control conditions in a growth chamber,as described above, and photographed.

**Enzyme assays:**Leaves of transgenic and wild-type plants were harvested after heat treatment as mentioned above and frozen immediately in liquid nitrogen. For analysis of ascorbate peroxidase (APX) and peroxidase (POD) activities, total soluble proteins were extracted from the leaves of plants with extraction buffer (for APX: 50 mM HEPES, pH 7.0, 0.1 mM EDTA; for POD: 100 mM potassium phosphate, pH 6.0), and protein concentrations were determined by the Bio-Rad protein assay kit. APX activity was determined by estimating the rate of ascorbate oxidation (extinction coefficient: 2.8 $mM^{-1}$ $cm^{-1}$) as described previously (Nakano and Asada, 1981). The oxidation of ascorbate was initiated by $H_2O_2$, and the decrease at 290 nm was monitored for 1.5 min. The reaction mixture contained 40 mM HEPES (pH 7.0), 0.1 mM EDTA, 0.03 mMAsA, 0.1 mM $H_2O_2$ and enzyme extract. One unit of APX activity was defined as the amount of enzyme that caused the oxidation of 1 mol of AsA. Similarly, POD activity was assayed according to the method described previously(Kwak *et al*., 1995). POD activity was measured by monitoring the increase in absorbance at 420 nm during the oxidation of pyrogallol in 20 second. The reaction mixture contained 10 mM potassium phosphate (pH 6.0), 7.8 mM $H_2O_2$, 0.5% pyrogallol and enzyme extract. One unit of POD was defined as the amount of enzyme that caused the formation of 1 mg of purpurogallin from pyrogallol.

## RESULTS AND DISCUSSION

**Production of transgenic creeping bentgrass plants over-expressing *MsHsp23*:**Small Hsps are most widely distributed and involves in the responses of various abiotic stresses among other Hsps (Sun *et al*., 2002). In additions, they play important roles in plant development (Dafny-Yelin*et. al*., 2008; Chauhan *et al*., 2012). It has also been reported that heat stress causes the induction of members of diverse sub-families of small Hsps in rice (Chen *et al*., 2014). However, among various subcellular-localized small Hsps, investigations of functional characteristics of mitochondrial small Hspsare largely unknown and remainelusive. Chaperone properties of small Hspshave been well characterized in their functions preventingthe denaturation of heat-injured proteins

(Siddique *et al*., 2008). To explore the potential of chaperone roles of small Hsp for enhancing the tolerance to heat stress (thermotolerance) in plants, we developed heterologous expression of alfalfa small Hsp23 (*MsHsp23*) in creeping bentgrass.We had previously described how alfalfa mitochondrial small *Hsp23* (*MsHsp23*), isolated by the GeneFishing technique, and its overexpression in tobacco and tall fescue plants enhanced tolerance to salinity and arsenic stress (Lee *et al*., 2012a,b). A construct harboring *MsHsp23*cDNA and a *HPT*gene driven by theCaMV 35S promoter was introduced into the creeping bentgrass genome by *Agrobacterium*-mediated transformation as described (Lee *et al*., 2011). Using the hygromycin-resistant screening, nine independent transgenic (Tg) lines were produced. Genomic DNAs from all transformants were obtained and PCR was analyzed using gene-specific primers to confirmthe insertion of transgenes. PCR analysis demonstrated the expression of *MsHsp23* in all Tg lines (Fig. 2A), whereas un-transformed plants lacked the signal (NT). Consistently, all Tg lines showed expression of the *HPT*gene by PCR amplification using gene-specific primers as described (Fig. 2B). Thus, all Tg lines were characterized by the presence and overexpression of *MsHsp23*.

All independent Tg lines were further confirmed for integration of *MsHsp23* using Southern blot analysis. Genomic DNA was digested by *Eco*RI, transferred to membrane and hybridized by gene-specific probes. As results of hybridization, all Tg lines showed diverse patterns of integration as well as copy numbers of inserted *MsHsp23* gene (Fig. 3). Based on Southern hybridization data, Tg-1 and Tg-2 were selected for further experiments because they showed the presence of one copy, while higher copy numbers werepresent in Tg-5, Tg-6, Tg-8 and Tg-9, respectively.

**Thermotolerance of creeping bentgrass overexpressing *MsHsp23*:**Selected Tg lines, Tg-1 and Tg-2, were transferred to soil in pots and grown for another 12weeks. As shown in Fig. 4A, Tg-1 and Tg-2 showed no phenotypic differences with non-transformed plants except for a somewhat smaller plant height in the Tg-1 line (Fig. 4A). Twelve weekold plants were exposed to heat stress conditions (42°C) for 24hand allowed to recovere for 7days. Both Tg lines (Tg-1 and Tg-2) overexpressing *MsHsp23* exhibited minor heat damage represented bya smaller amountof wilted leaves anda healthygreenish color, while the NT plants became largely wilted with light-green colored leaves(Figs. 4B and 4C). Extreme heat stress to living organisms causes the inactivation of diverse functional proteins and subcellular components in the cells. Leaf color is commonly changed as well, turning pale-green due to breakdown the chlorophyll by photo-oxidative damage induced by abiotic stresses (Feierabend and Winkelhüsener, 1982; Zlatev and Yordanov, 2004; Erge*et al*., 2008). Cytosolic and chloroplastic small Hspshave been investigated in their function promoting tolerance against heat and drought stresses (Sun *et al*., 2002; Murakami *et al*., 2004; Sato and Yokoya, 2008; Chauhan *et al*., 2012). However, abiotic stress defense phenotypes of mitochondrial small Hspsare largely unknown. Our data suggest that the overexpression of mitochondrial *MsHsp23* in creeping bentgrass enhances thermotolerance.

**Enhanced antioxidant enzyme activity in transgenic creeping bentgrass overexpressing *MsHsp23*:**Various abiotic stresses cause dramatic increase of reactive oxygen species (ROS) in plants. ROS levels are regulated by ROS generating and scavenging enzyme in the cytolplasm and various subcellular organelles, such as chloroplasts and mitochondria (Miller *et al*., 2010). Recently, the heterologous expression expression of rice SUMO E3 ligase (OsSIZ1) in creeping bentgrass, was shown to generally enhance abiotic stress tolerance, including heat tolerance. This was accompanied by strongly enhanced expression of small Hsps, such as Hsp16.5, Hsp26.7α and 26.8 (Li *et al*., 2013). This previous study also suggested that heterologous expression of MsHsp23 in tall fescue resulted in reduced accumulation of toxic oxygen radicals, such as hydrogen peroxide and thiobarbituric acid reactive substance (TBARS),following salinity and arsenic stress (Lee *et al*., 2012a). Thus, it indicates that overexpression of small Hsps in plants not only triggers their intrinsic chaperone properties to prevent stress-inducible substrate degradation, but also leads to the induction of antioxidant enzyme activities that will block increases of toxic ROS that typically accompany various stresses. To examine this induction of antioxidant enzymes by overexpression of *MsHsp23*, ascorbate peroxidase (APX) and peroxidase (POD) activities were assayed after heat treatments at 42 °C for 48 h. As shown in Fig. 5A, APX activity was enhanced by heat treatments in all lines including untransformed creeping bentgrass (NT), but the activity was significantly higher in Tg-2 compared to the activity in NTandTg-1 plants. In contrast, POD activity in NT plants was greatly enhanced by heat treatments, which was not observed in transgenic creeping bentgrass, neither in Tg-1 or Tg-2 (Fig. 5B). Antioxidant enzymes are widely distributed in different subcellular organelles.They exist in families of homologs with high numbers of copies (Miller *et al*., 2010). APX is found in both the chloroplast lumen and stroma, in peroxisomes, the cytosol and the inner membrane space of mitochondria. It effectively scavenges hydrogen peroxide to water (Miller *et al*., 2010). The overexpression of lily small Hsp16.45 in *Arabidopsis*was shown to enhance abiotic stress toleranceapparently based onenhanced activities of superoxide dismutase and catalase (Mu *et al*.,

2013).Althoughfunctional interactionsbetween small Hsps and antioxidant enzymes are still largely unknown, the overexpression of small Hspsseems to trigger enhanced antioxidant enzyme activities.

In summary, we have shown that heterologous expression of *MsHsp23*in creeping bentgrassprotects the plants against heat stress. Together with previous studies, MsHsp23 is involved in, or at the basis of, various abiotic stress responses, such as heat, salt and arsenic stresses. MsHsp23 heterologous overexpression in different plants confers dramatic stress tolerance accompanied by lower accumulation of ROS based on enhanced activities of antioxidant enzymes.


**Fig. 1.Schematic diagram of the T-DNA region of the pCAM-*MsHsp23* gene expression construct.**

The pCAM-MsHsp23 construct was used for transformation into calli of creeping bentgrass. RB, right border; LB, left border; 35S, CaMV 35S promoter; HPT, hygromycinphosphotransferase; MsHsp23, alfalfa small heat shock protein 23; T35S, CaMV 35S terminator; TNOS, nopaline synthase terminator. Arrows indicate the gene-specific primers for *HPT* and *MsHsp23*.

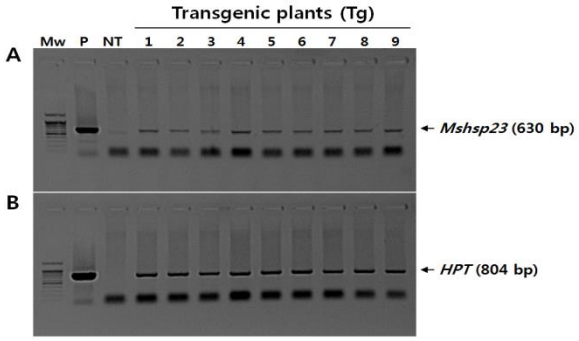


**Fig. 2.PCR analysis of transgenes in*MsHsp23* and *HPT* in creeping bentgrass.**

Transgenes, *MsHsp23* (A) and *HPT* (B) were amplified by PCR using gene-specific primers which described in Materials and Methods. Arrows indicate specific bands corresponding *MsHsp23* and *HPT* with 630 bp and 804 bp, respectively. Mw, molecular weight marker; P, pCAM-*MsHsp23* vector; NT, non-transformant creeping bentgrass plant; Tg, transgenic plants.

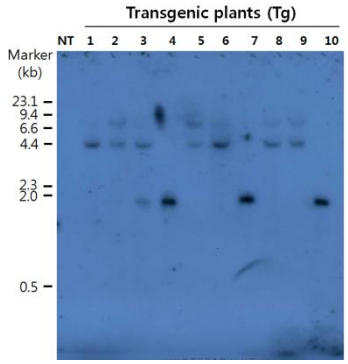


**Fig. 3.Southern blot analysis of *MsHsp23* in untransformed and transformed creeping bentgrass plants.**

Genomic DNA was extracted from non- (NT) and transformant (Tg) plants and digested by *Eco*RI restriction enzyme. Digested DNA was separated on 1 % agarose gel, transferred onto membrane, and hybridized with $^{32}$P-labelled *MsHsp23* probe as mentioned in Materials and Methods. Nucleotide size markers (Mw) represents in *left*.

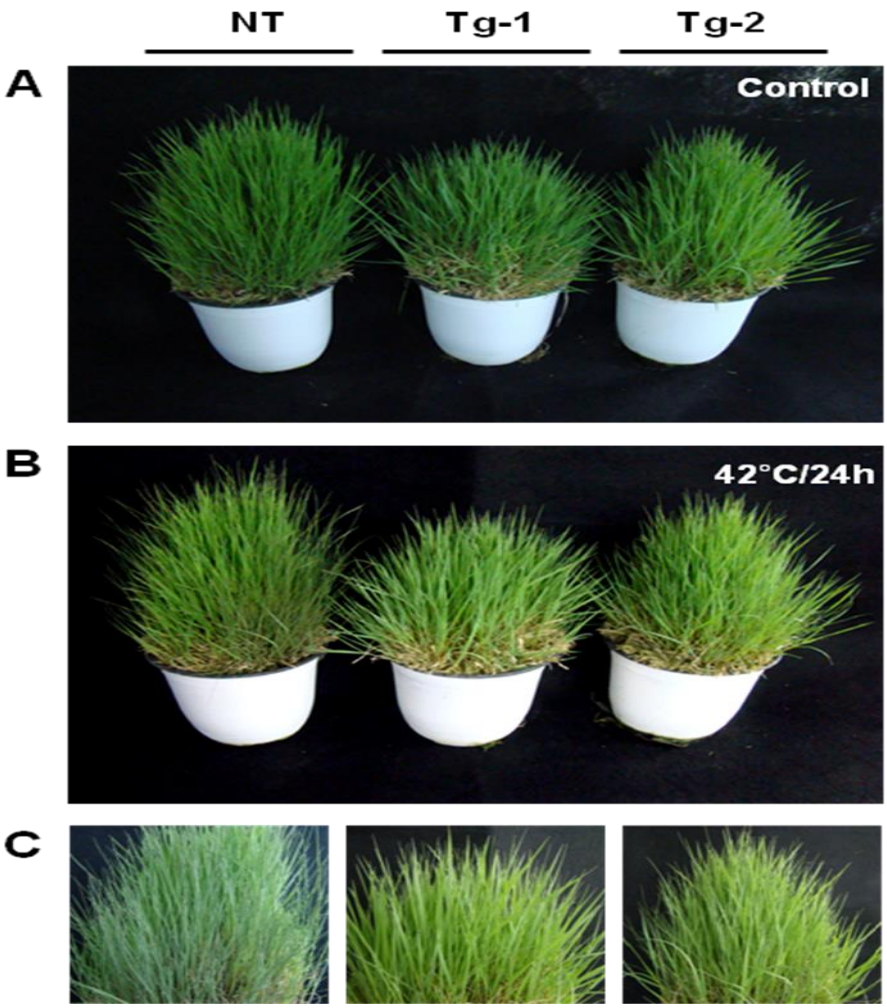


**Fig. 4.Thermotolerant assay of creeping bentgrass overexpressing *MsHsp23***

Non- (NT) and transformants (Tg-1 and Tg-2) were grown for 12weeks and exposed to 25°C (for control, A) or 42°C (for heat treatments, B) for 24h. C. The picture represents close-up of B. Both lines were illuminated identically.

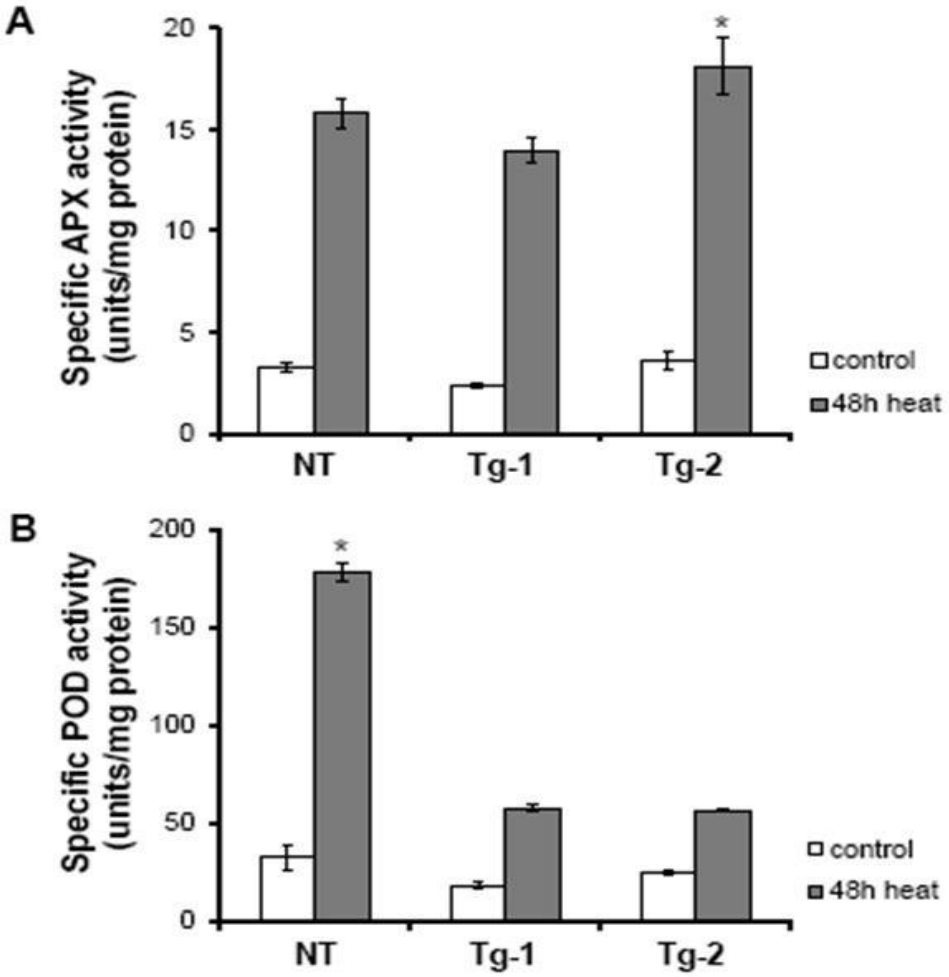


**Fig. 5.Antioxidant enzyme activities in response to heat stress.**

Leaves of untransformed (NT) and transformed (Tg-1 and Tg-2) plants were harvested after heat treatments (42°C) or at control temperature (25°C) for 48h. Activities of ascorbate peroxidase (APX, shown in A) and peroxidase (POD, B) were measured as outlined in materials and methods. Each assay was carried out with three independent replications. Bars represent the mean ± SE. Differences shown by asterisks are significant ($P<0.05$).

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