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Growth and Tuberization of Transgenic Potato Plants Expressing Sense and Antisense Sequences of Cu/Zn Superoxide Dismutase from Lily Chloroplasts

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Overexpression of a chloroplast-localized Cu/Zn superoxide dismutase (chCu/ZnSOD) obtained from lily significantly affects the growth and shape of potato tubers from an *in vitro* culture system (Kim et al., 2007). Here, we further characterized the sense and antisense transgenic potatoes grown and pots and the greenhouse to investigate the potential for more practical field applications of such phenotypic manipulations. Under *in vitro* conditions, antisense transgenic plants showed increased shoot growth, delayed tuberization, and altered tuber shapes. When antisense plants were treated with paclobutrazol, an inhibitor of GA biosynthesis, tuberization efficiency and tuber shape were recovered to a status very similar to that of *in vitro*-grown wild-type plants. Our results strongly support the idea that potato tuberization and shape is mediated by SOD-catalyzed reactive oxygen species, possibly via the GA biosynthesis pathway.

Keywords: antisense, Cu/Zn superoxide dismutase, gibberellin, sense, *Solanum tuberosum*, tuberization

Superoxide dismutase (SOD; superoxide: superoxide oxidoreductase, EC. 1.15.1.1: SOD) converts two superoxide anions (O'^{-}_{2}) into H_2O_2 and O_2. This enzyme is found in all oxygen-consuming organisms, aero-tolerant anaerobes, and some obligate anaerobes (Fink and Scandalios, 2002). Hence, it is regarded as the first component in an antioxidant defense system against damage from a reactive oxygen species (ROS) (Fridovich, 1991; Fink and Scandalios, 2002). So far, three types of SOD -- manganese SOD, iron SOD, and copper-zinc SOD (Cu/ZnSOD) (Fink and Scandalios, 2002) -- have been identified in plants, where they differ in their covalently linked catalytic metal ions and cellular locations.

The role of a ROS as a signaling molecule has been extensively studied because plants appear to deliberately regulate the amount of ROS in order to control various physiological processes, including pathogen defense (Levine et al., 1994; Alvarez et al., 1998), tolerance to abiotic stresses (Prasad et al., 1994), root development (Joo et al., 2001), senescence (Zimmermann and Zentgraf, 2005), and stomatal behavior (McAinsh et al., 1996).

In the early stages of pathogen infection, several ROS can be produced as a result of the enhanced enzymatic activity of plasma membrane-bound NADPH-oxidase, cell wall-bound peroxidase, and amine oxidases in the apoplasts (Apel and Hirt, 2004). Among these, H_2O_2, produced from an oxidative burst by pathogen attack, orchestrates a localized hypersensitive response and functions as a secondary messenger, mediating the systemic expression of various defense-related genes (Levine et al., 1994; Orozco-Cardenas and Ryan, 1999). In fact, transgenic potato plants expressing high levels of H_2O_2 are reported to be more resistant to pathogen attack (Wu et al., 1997).

In response to environmental stresses, e.g., drought and low temperatures, the accumulation of ROS, including H_2O_2, is proposed as the first step in signal transduction (Shinozaki and Yamaguchi-Shinozaki, 1997). Both H_2O_2 and menadione (superoxide-generating compound) induce cold tolerance in chilling-sensitive maize seedlings (Prasad et al., 1994). Gravity also induces asymmetric ROS generation in roots, and the scavenging of ROS inhibits maize root gravitropism, clearly showing that ROS is a key determinant of such behavior (Joo et al., 2001). ROS is also involved in senescence, in which the peroxidation of lipids can be triggered by ROS that is generated by the action of lipoxygenase (Zimmermann and Zentgraf, 2005). Both H_2O_2 and methyl viologen, the latter generating superoxide anions, inhibit the opening of the stomata but promote their closure (McAinsh et al., 1996).

Gibberellins (GAs) are tetracyclic diterpenoid hormones that regulate plant growth and development. In potato, GAs inhibit tuberization, as shown by the strong negative correlation between GA levels and tuber initiation (Hussey and Stacey, 1984; Jeon et al., 1992; Jackson and Prat, 1996). However, the mechanism by which this is accomplished remains unknown. Tuberization involves a shift in the growth of a stolon, from extension to radial development. In potato, tubers usually arise from basal stolons through marked radial enlargement of the stem axis basipetal to the stolon apex. The length of a tuber is defined as the distance between the apex (rose) and the place of stolon attachment (heel). New tubers are recognizable by their swollen subapical region (>2 mm) that is more than twice the diameter of the rest of the stolon.

We previously isolated a chloroplast-localized Cu/ZnSOD (chCu/ZnSOD) from lily during bulb formation, then intro-

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Abbreviations: chCu/ZnSOD, chloroplast-localized Cu/ZnSOD; ROS, reactive oxygen species

duced it into the potato. In addition to changes in their scavenging capacity for oxidative stress, those sense- and antisense-transgenic potato plants show different morphological characteristics and growth patterns during tissue culture (Park et al., 2006). In a vial culture system, we have easily detected the contrasting effects of plant growth between the overexpressing sense line (SS4) and the antisense line (SA1). Both show increased ROS concentrations compared with the wild-type plants. Whereas overexpression of chCu/ZnSOD primarily increases H_2O_2 contents, higher levels of the superoxide anion are detected in SA1. Interestingly, greater concentrations of GAs are measured during plant growth and the early stage of tuberization in SA1. Therefore, we have previously suggested that the superoxide anion acts as a signal transducer via GA biosynthetic pathways for the regulation of plant growth and tuber development in potato (Kim et al., 2007).

In the present study, we further explored this hypothesis by correlating the changes in GA levels with phenotype differences between the sense and antisense lily chCu/ZnSOD-transgenic potatoes when grown in pots and a greenhouse. Here, we report a newly identified physiological role for chCu/ZnSOD in potato tuber development, as regulated by a GA biosynthesis pathway.

MATERIALS AND METHODS

Plant Material and Growing Conditions

We used wild-type (WT) plants as well as sense and antisense transgenic plants of *Solanum tuberosum* L. cv. Desiree that express chCu/ZnSOD sequences from lily (Park et al., 2006). Sense and antisense lily chCu/ZnSOD cDNA, under the CaMV35S promoter in PMBP vectors, were transformed into *Agrobacterium tumefaciens* strain LBA4404, then introduced into 'Desiree' plants via leaf disc transformation. These transgenic potato lines were cultured in a growth room maintained at 25 ± 1°C under a 16-h photoperiod from fluorescent lamps (Osrams; 50 μmol photons m^{-2} s^{-1}). Microtubers were produced as previously described (Jeon et al., 1992), with minor modifications. Nodal cuttings taken from shoot cultures were placed on the medium as stated above, but the amount of supplemental sucrose was increased to 90 g L^{-1}. For our reversal test, tuberization was observed for 7 d after tubers were induced by treatment with paclobutrazol (PBZ; 0.17 mM) or GA_3 (0.29 μM). The growth room for this *in vitro* procedure was maintained at 20°C. The resultant microtubers were then grown in a greenhouse in pots, and plant growth and yields were recorded. Tuber shapes were phenotyped by the ratio of length to width (L/W), as described by Lisinska and Leszczynski (1989).

Determination of Net O_2 Evolution and Net O_2 Uptake

Leaf discs were excised and introduced into an O_2 electrode chamber (Hansatech Instruments, UK), using a closed gas exchange system of air with 5% CO_2 at 26 ± 2°C, as described previously (Choi et al., 2002). After calibration, the steady-state rate of O_2 consumption was monitored until a stable rate was reached. The discs were then illuminated at an irradiance of 900 μmol m^{-2} s^{-1}. Actinic light was provided by a 150 W quartz-halogen slide projector fitted with heat filters through neutral density filters.

RESULTS AND DISCUSSION

Plants respond to changes in their environment by deliberately varying the amounts of ROS that deliver environmental signals to the nucleus and transcribe specific genes. Because of their effectiveness as signal transducers and their harmful effects when produced in excess, ROS concentrations are subject to regulation in a highly sophisticated manner (Schopfer et al., 2001; Apel and Hirt, 2004). As expected, our transgenic potato lines containing the sense orientation (Line SS4) of lily chCu/ZnSOD exhibited increased tolerance to oxidative stresses, such as the herbicide methyl viologen (MV), whereas the antisense lines (SA1) had decreased tolerance (Park et al., 2006).

Interestingly, we found differences in microtuberization and plant morphological characteristics between the WT and transgenics when they were maintained in a mass production system. Under vial-culturing system, the contrasting effects on plant growth were apparent between lines SS4 and SA1. Additionally, greater concentrations of H_2O_2 were detected in SS4 while higher levels of the superoxide anion were detected in SA1 than in the WT. Therefore, we conclude that this increased amount of the superoxide anion may act as a signal transducer for regulating potato plant growth and tuber development by acting on the GA biosynthetic pathways (Kim et al., 2007).

To confirm this, we noted the *in situ* phenotypic characteristics of microtubers from WT and transgenic potato plants grown in pots (Fig. 1A, B). Stem growth of the antisense transgenics (SA1 and SA2) was 2- to 2.5-fold higher than for either the WT or the sense transgenics (SS3 and SS4). This dramatic increase was probably related to photosynthetic and respiratory activities. Therefore, we analyzed those processes in leaf discs (Fig. 1C). Dark respiratory O_2 uptake and maximal photosynthetic O_2 evolution rates in the SA1 and SA2 lines were not significantly affected by antisense expression of lily chCu/ZnSOD, thus ruling out the idea that increased height growth is not mainly regulated by energy metabolism. We also investigated whether stem elongation was primarily controlled by GA levels. As expected, when our antisense plants were treated with the GA biosynthesis inhibitor PBZ, their heights were reduced, with measured values similar to the WT. Likewise, when GA_3 was applied to the overexpressing SS4 plants, their stems were elongated to heights close to those of the SA1 plants (data not shown). This again suggests the involvement of GA in SOD-induced accelerated stem growth.

GAs function via cell elongation (Graebe, 1987). This class of growth hormone also significantly inhibits tuber formation and reverses the process of *in vitro* tuberization at high concentrations, while, at low concentrations, it promotes tuber growth in the later stages of tuberization (Kim et al., 2005). We have previously shown that *in vitro* tuberization is delayed in antisense plants but promoted in overexpressing

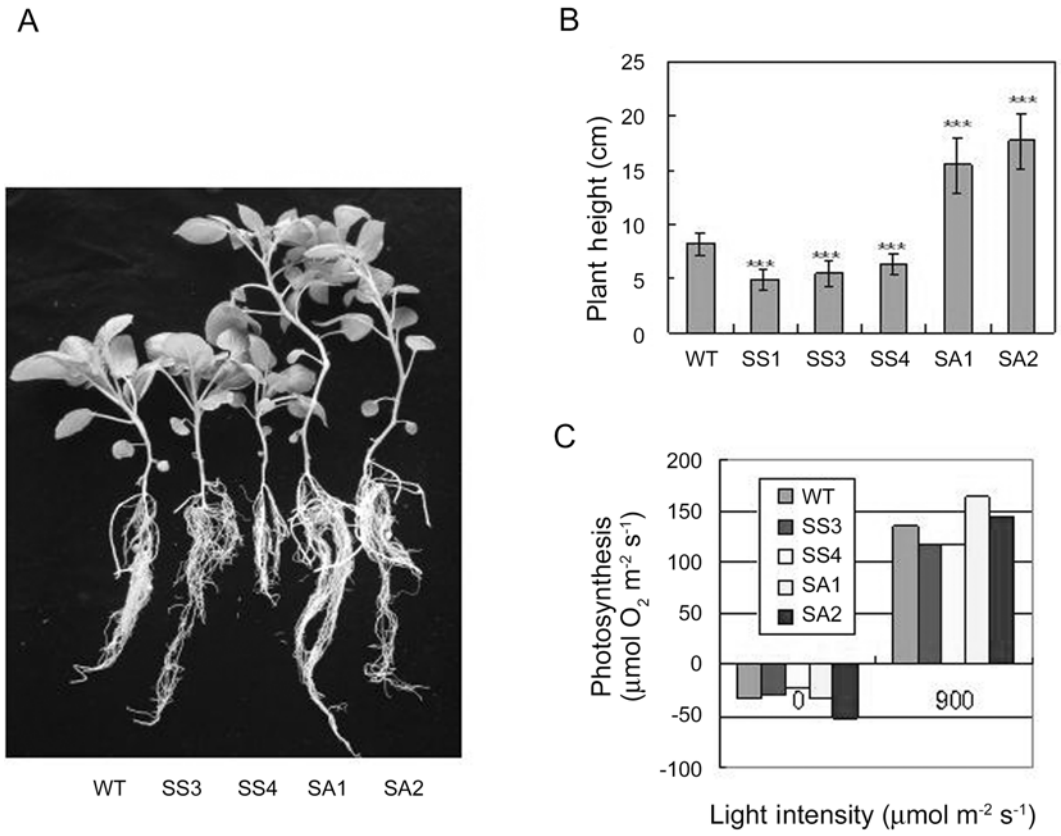

Figure 1. Photograph (**A**), plant heights (**B**), and rates of respiration and photosynthesis (**C**) for wild types (WT) and transgenic plants (SA and SS) of potato (*S. tuberosum* cv. Desiree) grown in pots for 4 weeks under greenhouse conditions. Lines SS3 and 4 are lily chCu/ZnSOD sense strand transgenics; Lines SA1 and 2 are lily chCu/ZnSOD antisense strand transgenics. Data are from three replicate experiments. In (**B**) error bars represent SD (n=30 or greater). In (**C**) error bars represent SD (n=3 or 4). Three asterisks indicate significant difference in mean values between WT and transgenic plants at $P < 0.001$, as determined by ANOVA *t*-test (n=30). Error bars indicate mean ± SD.

SS4 plants (Kim et al., 2007). The normal microtuberization pattern can be completely restored, however, following treatment with GA_3 or PBZ (Kim et al., 2005, 2007).

Therefore, to verify that the changes in phenotype and tuberization patterns for the transgenics were mainly caused by active GA, we used an *in vitro* system to record the trends in microtuberization and tuber shape after treatment with GA_3 or paclobutrazol. Tuber formation was slower from SA1 than from either WT or SS4 (Fig. 2A), possibly because of the higher concentrations of GAs found in the SA1 plants. PBZ applications completely restored tuberization frequency. In SS4 plants, such promotion was thoroughly delayed by GA_3 (Fig. 2A). Tuber shapes differed slightly between the *in vitro* and greenhouse systems but, generally, the shift to a more elongated form was the same in the SA1 plants. Microtubers of WT and SS4 were round or oval, whereas those of SA1 were mostly oval (Fig. 2B, C). PBZ treatment also completely restored the latter form to a round, oval shape. Likewise, the round, oval-shaped microtubers of WT and SS4 were also changed into oblong forms through treatment with GA_3 (Fig. 2C).

Compounds with a nitrogen-containing heterocycle, such as triazoles and imidazoles, act as inhibitors of various P450-dependent reactions. Among them, paclobutrazol and uniconazole are utilized as plant growth retardants (Rademacher, 2000). These chemicals block P450 *ent*-kaurene oxidase (CYP701), which catalyzes the oxidation of *ent*-kaurene to *ent*-kaurenoic acid in GA biosynthesis (Burden et al., 1987). In addition, they may interfere with brassinosteroid biosynthesis and be potent inhibitors of ABA catabolism in *Arabidopsis* (Asami et al., 2001; Saito et al., 2006). Therefore, it is possible that paclobutrazol may have inhibited not only GA_3 but also other hormones in the SA1 plants. However, for SS4, application of GA_3 completely restored tuber growth and shape. Therefore, we might suggest that changes in phenotypes and tuberization patterns of the transgenics are mainly caused by active GA levels.

We also raised transgenic potato plants in a greenhouse environment that simulated field conditions. SA1 lines grew significantly faster than the WT and SS4 lines (Fig. 3A), confirming our earlier results from both pot- (Fig. 1A) and *in vitro*-culturing (Kim et al., 2007). Compared with the WT, growth rates for SA1 were 45, 81, and 44% higher at 3, 5, and 7 weeks, respectively. In contrast, SS4 grew significantly slower after 3 weeks, compared with the WT (data not shown). Tuber shapes and yields from the sense and antisense transgenics also differed (Fig. 3C, D). Their development was recorded for both greenhouse and Petri dish plants. Shapes were characterized by the ratio of length to width (L divided by W) (Lisinska and Leszczynski, 1989), and followed these classifications: round-shortened, <0.9; round, 0.9 to 1.2; round-oval, 1.2 to 1.6; oval, 1.6 to 1.8;

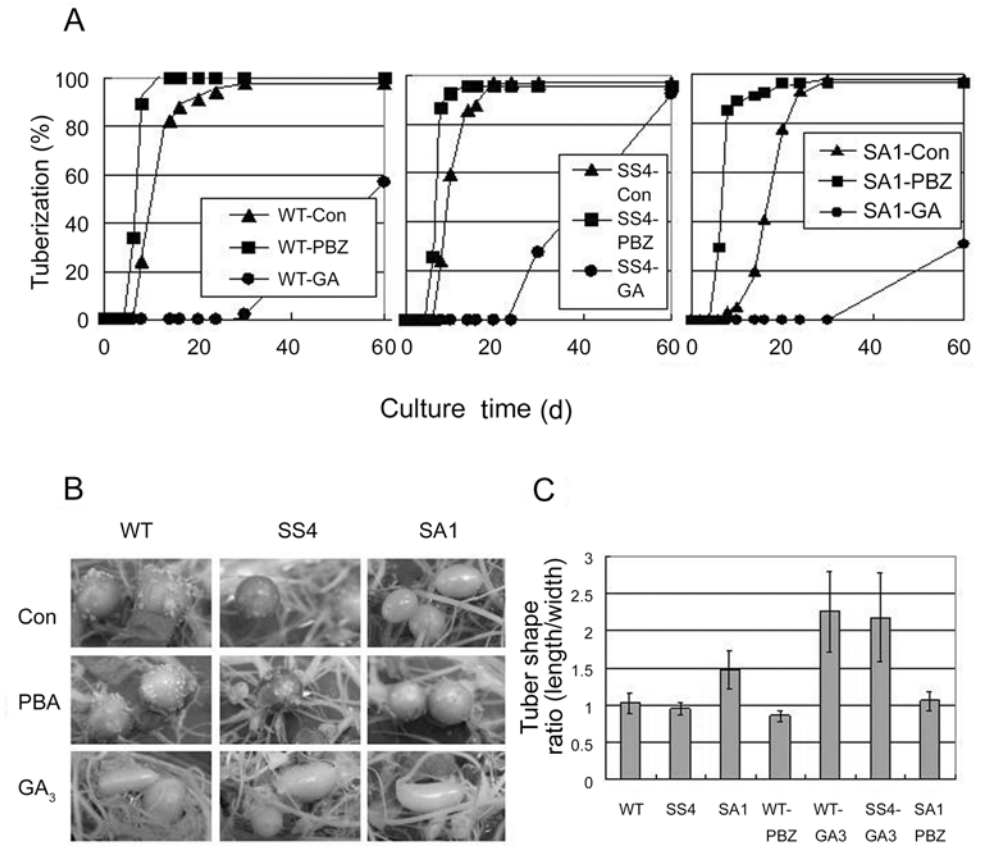

Figure 2. Tuberization (**A**), photograph (**B**) and tuber shape (**C**) of wild types (WT) and transgenic plants (SA and SS) of potato (*S. tuberosum* cv. Desiree) during *in vitro* tuberization from nodal explants. Microtuberization was investigated for 60 d after tubers were induced by treatment with PBZ (0.17 mM) or GA_3 (0.29 mM). Nodal explants were cultured, 13 per Petri dish, at 20°C, with 7 replicates. In (**C**) tuber shapes were defined by ratio of length to width (L/W) for the every 30 microtubers. Mean values are presented with standard deviation.

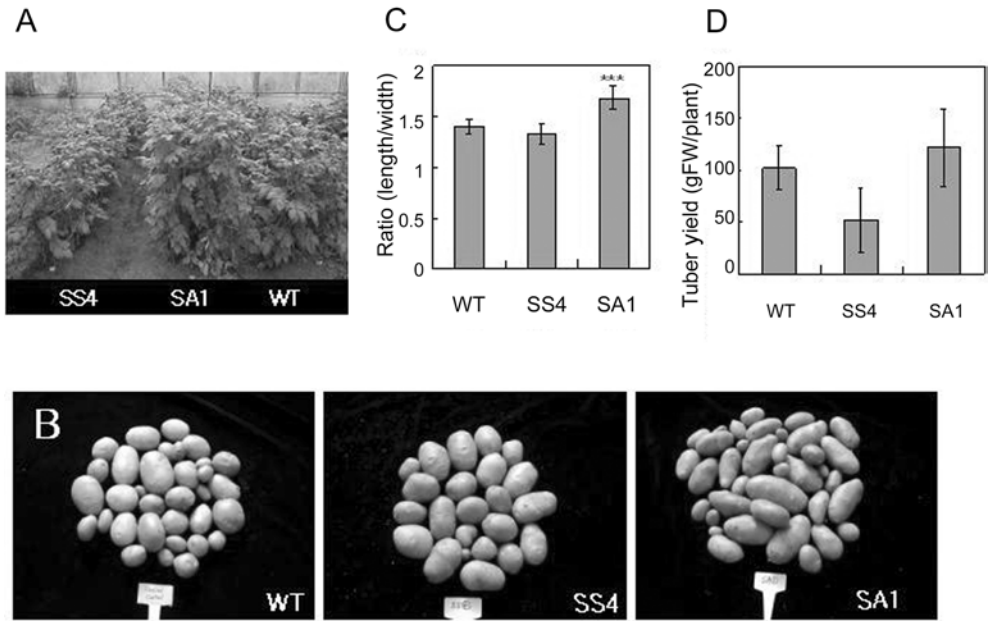

Figure 3. Photograph of plants (**A**) and tuber (**B**), tuber shape (**C**), and yield (**D**) from wild types (WT) and transgenic lines (SA and SS) of potato (*S. tuberosum* cv. Desiree) grown in soil under greenhouse conditions for 7 weeks. Lines SS4 are lily chCu/ZnSOD sense strand transgenic; Lines SA1 are lily chCu/ZnSOD antisense strand trasnsgenic. Three asterisks indicate significant difference in mean values between WT and transgenic plants at $P < 0.001$, as determined by the ANOVA *t*-test (n=30). Error bars indicate mean ± SD.

oval-oblong, 1.8 to 2.0; and oblong, >2.0. Line SA1 (L/W 1.69; oval) had a significantly higher ratio than SS4 (L/W 1.33; round-oval) and WT (L/W 1.4; round-oval) (Fig. 3B, C). With regard to yields from greenhouse-grown plants, SA1 showed a 19.6% increase in tuber weight (122 g FW per plant) while SS4 was 41% lower (52 g FW per plant) compared with the WT (102 g FW per plant).

To effectively regulate plant growth and metabolism, ROS should be interconnected with other signaling pathways. Indeed, Cui et al. (1999) and Joo et al. (2001) have described the important relationship between ROS and plant hormones. ROS generated in the chloroplasts influence a broad range of the nuclear transcriptome (Vranova, 2002), which might suggest that a change in nuclear gene expression in SA1 produces higher concentrations of the superoxide anion. For example, when tobacco plants are exposed to MV, an inducer of oxidative stress, at least 95 out of approximately 5370 genes are altered. We previously detected the up-regulation of the P450 *ent*-kaurene *oxidase* gene in SA1 plants, which contain higher levels of superoxide anion than do the WT; this enhanced expression contributes to the increased amount of GAs (Kim et al., 2007). P450 *ent*-kaurene oxidase, a gene in the synthesis pathway for GAs, is transcribed in the nucleus and transported to the chloroplasts. Our current findings strongly suggest that the GA level is modulated by the types and concentration of ROS that rely on chCu/ZnSOD in potato. Accordingly, gibberellin affects stem growth, tuberization, and tuber shape. Among the various ROS, the superoxide anion is likely responsible for GA content because it is significantly increased in the antisense plants (Kim et al., 2007).

Information about the role of particular ROS would enable researchers to develop a practical approach using chCu/ZnSOD antisense transgenics to control potato plant growth and tuber shape. The latter feature, which is largely variety-specific, is dominated by a round rather than a long shape (Howard, 1970). Breeder selection is determined by customer preferences and requirements of the processing industry. For example, to minimize waste, varieties with long tubers are preferred for French fries, while those with round tubers are better for crisps. Therefore, we can use these data to develop transgenic potatoes where shape can be manipulated to accommodate individual needs.

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