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RESEARCH ARTICLE

Cloning and prokaryotic expression of translationally controlled tumor protein (*TaTCTP*) gene from wheat and preparation of antiserum

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Abstract **The translationally controlled tumor protein (TCTP) is a multi-functioning protein that performs vital roles, particularly in various complicated life processes. To further understand the biological function of translationally controlled tumor protein (*TaTCTP*) gene from wheat, total RNA was isolated from wheat leaves and then *TaTCTP* gene was amplified by PCR after reverse transcription by using the anchored primers oligo(dT)$_{18}$. And then *TaTCTP* gene was connected into PMD19-T vector for sequence. The recombinant expression vector (pET30a-GST-*TaTCTP*-His) was constructed and transformed into *E. coli* strain Rosetta (DE3) subsequently, then a proximate 20 kDa protein in Rosetta (DE3) was expressed and characterized by SDS-PAGE. Moreover, TaTCTP fusion protein was purified by Ni-NTA affinity chromatography from recombinant bacterial lysate and was used to immunize rabbit to produce polyclonal antibody. The titer and specificity of the anti-TaTCTP antibody were successfully detected by indirect ELISA and Western blot analysis.**

Keywords translationally controlled tumor protein (TCTP), wheat, prokaryotic expression, Western blot analysis

Introduction

The translationally controlled tumor protein (TCTP) was first identified in Ehrlich ascites tumor cell lines in the 1980s (Böhm et al., 1989). Subsequently, it was found that TCTP was not tumor specific but ubiquitously present in all normal types of mammal cells except kidney cells (Sanchez et al., 1997). Among them, the mouse TCTP and human TCTP were known as P21 and P23, respectively (Chitpatima et al., 1988; Gross et al., 1989). The gene encoding protein is tumor protein translationally-controlled 1 (*TPT1*). With further researches, the homologs of *TCTP* were identified in different eukaryotes, including plants, animals and yeasts (Thaw et al., 2001). *TCTP* encoded a hydrophilic protein of 18–23 kDa that showed no sequence similarity with any other known proteins (Susini et al., 2008). It suggested that TCTP is a highly conserved protein during evolution, which is widely expressed in all eukaryotic organisms and shows no significant sequence homology with any other protein families.

TCTP was initially identified as a growth-related protein. Recently, some studies suggested that TCTP may have extremely important biological functions in various biological processes, with domains involved in calcium binding (Kim et al., 2000) and microtubule stabilization (Yarm, 2002) and important for cell cycle progression, malignant transformation and tumor reversion. Kim et al. (2009) identified TCTP as a cytoplasmic repressor of Na, K-ATPase and confirmed that systemic hypertension was induced in transgenic mice overexpressing *TCTP* through inhibition of vascular Na, K-ATPase and increased in intracellular calcium mobilization. Graidist et al. (2007) reported that fortilin (also known as TCTP) is an intracellular Ca^{2+} scavenger, protecting cells against Ca^{2+}-dependent apoptosis by binding and sequestering Ca^{2+} from the downstream Ca^{2+}-dependent apoptotic pathways. Nagano-Ito et al. (2009) showed TCTP can prevent hydrogen peroxide-induced cell death by using retroviral-mediated expression cloning to identify cDNAs that inhibit cell death induced by oxidative stress. Expression

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of *TCTP* is highly sensitive to a wide range of extracellular stimuli that are involved in cell cycle control (Cans et al., 2003) and stress responses like calcium, heavy metals, heat shock and starvation, etc. (Bonnet et al., 2000). This protein is also implicated in many other functions such as inducing interleukin production (Bheekha-Escura et al., 2000), histamine releasing (MacDonald et al., 2001), and preventing apoptosis in nature (Liu et al., 2005). However, the primary biological function of TCTP is still unclear (Rao et al., 2002).

Currently, the reports about TCTP mainly focused on humans and animals, but very few researches were conducted with plants. Pay et al. (1992) first reported that *TCTP* existed in alfalfa (*Medicago sativa*). Sage-Ono et al. (1998) found that the level of *TCTP* was gradually increased during darkness in the morning glory (*Pharbitis nil*); Currently, *TCTP* genes had been isolated and cloned from pumpkin (*Cucurbita maxima*, DQ304537), rubber tree (*Hevea brasiliensis*, AF091455), *Arabidopsis* (*Arabidopsis thaliana*, NM112537), strawberry (*Fragaria×ananassa*, Z86091), Alfalfa (*Medicago sativa*, X98618), watermelon (*Citrullus lanatus*, AB182927), wheat (*Triticum aestivum*, AF508970), cabbage (*Brassica oleracea*, AF418663), salvia (*Salvia miltiorrhiza*, EF667003) and other plants. This work laid a foundation for further studying of the TCTP function in plants.

In our laboratory, Yan et al. (2009) obtained a differentially expressed fragment by the suppression subtractive hybridization in the wheat-leaf rust fungus interaction system. BlastX analysis showed that the deduced amino acid sequence was consistent with that of the wheat translationally controlled tumor protein (GenBank: AF508970.1). It suggested that TCTP may be involved in the process of wheat disease resistance. To investigate its location, expression changes and physiologic functions of TCTP in the defense responses to wheat leaf rust infection, the preparation of antiserum was very important and necessary. In this paper, we constructed prokaryotic expression vector, successfully expressed and purified fusion protein, and prepared antibodies by injecting the purified fusion protein TaTCTP into immune rabbits. It provided essential materials for further researches of TCTP functions.

Materials and methods

Materials

E. coli strain DH5α, Rosetta (DE3) and expression vector pET30a-GST were provided by the plant stress laboratory, Agricultural University of Hebei. pMD19-T vector, *Eco*RI, *Xho*I and T4 ligase were purchased from TAKARA Company. IPTG (Isopropyl-β-D-thiogalactopyranoside) and X-gal (5-bromo-4-chloro-3-indolylβ-D-galactopyranoside) were purchased from Shanghai Sangon Biotechnology Company.

RNA extraction

Total RNA was extracted from the leaf tissue of wheat near-isogenic line Tc*Lr19* following instruction of Takara RNAiso^{TM} Plus. The concentration of RNA was measured by NanoDrop ND-1000 (American) and the integrity of RNA was detected by 1% agarose gel electrophoresis.

The amplification of *TaTCTP* gene by PCR reaction

Total RNA was reverse-transcribed into first-strand cDNA using Reverse Transcriptase M-MLV (TAKARA D2640A) according to the manufacturer's instructions. 100 μL reaction volume was established using first-strand cDNA as template to carry out PCR reactions. Thermal cycling was performed for an initial denaturation at 94°C for 10 min followed by 35 cycles at 94°C for 30 s, 67°C for 45 s and 72°C for 45 s. The final extension was at 72°C for 10 min. The primers used with the underlined regions noted as restriction enzyme sites for *Eco*RI and *Xho*I are as follows:

TaTCTP:

F 5′-CCGGAATTCCAAGGAGCAGTTGATGTGG3′,

R 5′-CCCTCGAGGCACTTGACCTCTTTCAGCCCA3′.

Construction of expression vector

TCTP cDNA was gel-purified and connected into the pMD19-T vector, designated as pMD19-T-*TaTCTP*. Competent cells *Ecoli* DH5α was transferred into the pMD19-T-*TaTCTP* vector and selected on LB medium with IPTG and X-gal. The white colonies were picked out separately for the further identification by DNA sequencing. TCTP cDNA was gel-purified after the pMD19-T-*TaTCTP* vector was digested with *Eco*RI and *Xho*I, and recloned into pET30a-GST expression vector, designated as pET30a-GST-*TaTCTP*-His. After that, the expression vector was digested by *Eco*RI and *Xho*I to test.

Expression of TaTCTP protein in *E. coli* Roseta (DE3)

The recombinant vector pET30a-GST-*TaTCTP*-His was transformed into *E. coli* Roseta (DE3). To optimize its expression, the bacterial culture was grown at 37°C until OD_{600} of 0.5–0.8, at which time protein expression was induced by adding 0.1 mmol/L, 0.4 mmol/L, 0.8 mmol/L and 1.0 mmol/L IPTG, respectively. Then the culture was shaken at 200 r/min at 28°C in 100 mL erlenmeyer flask. After induction, the samples were taken at different time points. The bacteria were harvested by centrifugation at 8000 r/min for 15 min at 4°C, resuspended in buffer (10 mmol/L Tris-HCl, pH 8.0, and 100 mmol/L DTT) and disrupted by ultrasonication. After centrifugation, the pellet was resuspended in buffer. The supernatant and the resuspended pellet were analyzed by SDS-PAGE.

Protein purification and concentration detection

The TaTCTP fusion protein with His-tagged was purified by Ni-NTA affinity chromatography. Several tubes were added 100 μL Ni-NTA and 1.8 mL bacterial lysates, incubated overnight at 4°C. The next day, the tubes were centrifuged at 2600 r/min for 5min at 4°C, in which the supernatant was carefully removed. The Ni-NTA was washed three times with Column Buffer (20 mmol/L Tris-HCl, pH 7.4, 200 mmol/L NaCl and 1 mmol/L EDTA), then washed twice with 15 mmol/L imidazole, and then eluted with 300 mmol/L imidazole and analyzed by SDS-PAGE. The concentration of purified proteins was detected by Coomassie brilliant blue G-250. The purified protein was added to a final concentration of 10% glycerol and stored at −80°C.

Antiserum preparation using purified TaTCTP

The purified fusion protein TaTCTP as antigen was injected into rabbits to produce a polyclonal TaTCTP antibody. The concrete experimental protocol referred to Liu et al. (2010). The serum was collected and stored at −20°C for ELISA, Western blot, and immunohistochemistry assays.

Results

PCR amplification

Total RNA was analyzed by 1% (w/v) agarose gel electrophoresis to test the extraction quality (Fig. 1). The 28S rRNA had equal brightness with 18S rRNA in total RNA, which means the RNA had not degraded. The ORF of *TaTCTP* about 504 bp with the addition of *Eco*RI and *Xho*I restriction sites on both ends was successfully amplified with RT-PCR. The *TaTCTP* cDNA was analyzed by 1% (w/v) agarose gel electrophoresis (Fig. 2).

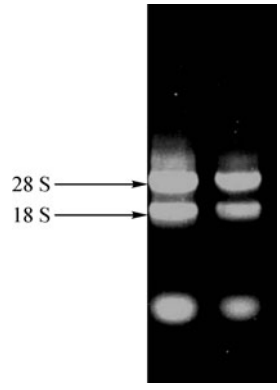

Figure 1 Integrity detection of total RNA of wheat leaves.

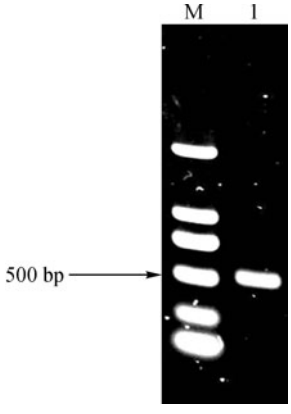

Figure 2 Amplification of *TaTCTP* gene. M is DNA marker; 1 means *TaTCTP* fragment.

Construction of recombinant pET30a-GST-TaTCTP-His and restriction enzyme digestion

The *TaTCTP* cDNA was cloned into pMD19-T. After the pMD19-T-*TaTCTP* vector was digested by *Eco*RI and *Xho*II, the fragment was gel-purified and reconstructed to obtain the recombinant plasmid pET30a-GST-*TaTCTP*-His. Its product was transformed into *E. coli* DH5α and subsequently sequenced. As a result, pET30a-GST-*TaTCTP*-His was detected by *Eco*RI and *Xho*II digestion and analyzed by 1% (w/v) agarose gel electrophoresis (Fig. 3).

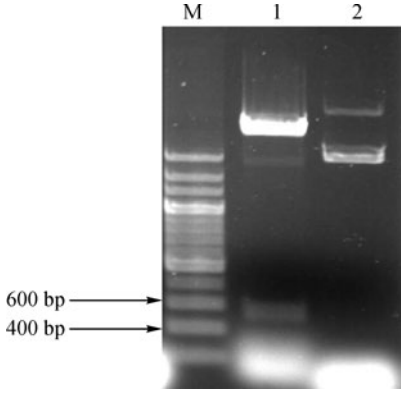

Figure 3 Restriction enzyme analysis of pET30a-GST-*TaTCTP*-His recombinant plasmid. M is DNA marker; 1 means digestion of pET30a-GST-*TaTCTP*-His recombinant plasmid with *Eco*RI and *Xho*II; 2 means the integrated pET30a-GST-*TaTCTP*-His.

Exploration of prokaryotic expression conditions of TaTCTP and detection of SDS-PAGE

The PET30a-GST-*TaTCTP*-His vector was transformed into *E. coli* Rosetta (DE3) cells. The bacterium liquid was incubated (at 37°C and 200 r/min) to an OD_{600} of 0.5–0.8, and then IPTG was added to a final concentration of 0.1 mmol/L, 0.4 mmol/L, 0.8 mmol/L and 1.0 mmol/L, respectively. After induction, the samples were taken at different time to detect the expression of fusion protein by

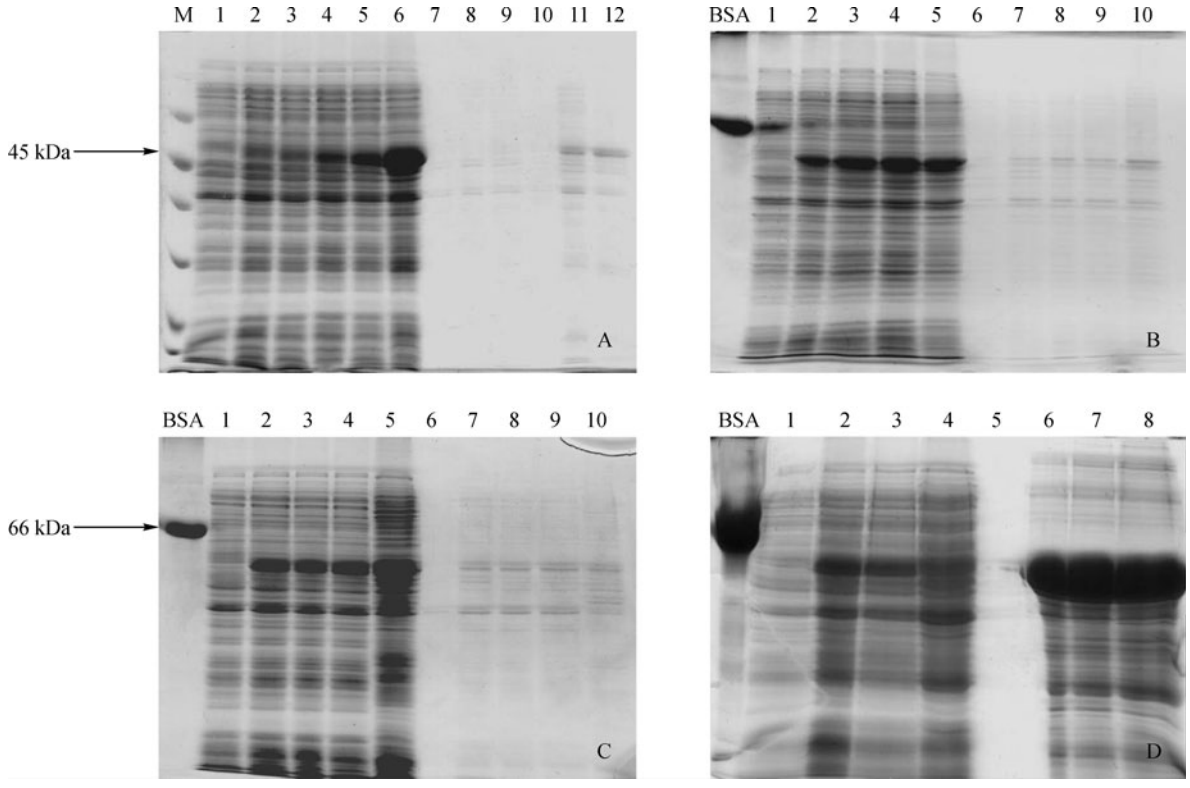

Figure 4 Exploration of prokaryotic expression conditions of TaTCTP. A: 1-6 mean supernatant, induced at 0 h, 1 h, 2 h, 4 h, 6 h and 20 h by 0.1 mmol/L IPTG treatment, respectively; 7–12 mean precipitate induced at 0 h, 1 h, 2 h, 4 h, 6 h and 20 h by 0.1 mmol/L IPTG, respectively. B: 1–5 mean supernatant induced at 0 h, 2 h, 4 h, 6 h and 20 h by 0.4 mmol/L IPTG treatment, respectively; 6–10 mean precipitate induced at 0 h, 2 h, 4 h, 6 h and 20 h by 0.4 mmol/L IPTG, respectively. C: 1–5 mean supernatant induced at 0 h, 2 h, 4 h, 6 h and 20 h by 0.8 mmol/L IPTG treatment, respectively; 6–10 mean precipitate induced at 0 h, 2 h, 4 h, 6 h and 20 h by 0.8 mmol/L IPTG, respectively. D: 1–4 mean supernatant induced at 0 h, 2 h, 4 h and 20 h by 1.0 mmol/L IPTG treatment, respectively; 5–8 mean precipitate induced at 0 h, 2 h, 4 h and 20 h by 1.0 mmol/L IPTG, respectively.

SDS-PAGE analysis. As shown in Fig. 4, an about 50 kDa band appeared, which was consistent with the theoretical size of fusion protein. The fusion protein was mainly in the supernatant at 0.1 mmol/L, 0.4 mmol/L, 0.8 mmol/L IPTG, while at 1 mmol/L IPTG, the fusion protein was present in the precipitate with the form of inclusion bodies. From the Fig. 4, we come to a conclusion that the conditions were induced for 20 h by 0.1 mmol/L IPTG with the highest expression level, which was identified as the optimal expression condition.

The purification of TaTCTP protein

The fusion protein TaTCTP was purified by Ni-NTA affinity chromatography. Bacterial lysis supernatant (before purification) and the eluent (after purification) were analyzed by SDS-PAGE. As shown in Fig. 5, the purified product only had one clear main band and the size of the purified protein was in accordance with the product of specific expression. Wash buffer removed most of the hybrid protein. After treated by elution buffer for a longer time, a large number of the fusion proteins were collected. The concentration of separated protein was 1.3 mg/mL, measured by Coomassie brilliant blue G-250.

Preparation and identification of polyclonal antiserum against TaTCTP

The New Zealand rabbits were immunized with the purified fusion protein TaTCTP. The titer of TaTCTP antiserum detected by indirect ELISA was 1:768000 (Fig. 6). Western blotting was used to detect the purified fusion protein and the whole wheat protein. The results showed that TaTCTP rabbit antiserum not only positively reacted with the purified fusion protein but also with the wheat whole protein (Fig. 7), indicating that the prepared antiserum has good specificity.

Discussion

The translationally controlled tumor protein (TCTP) as a conserved protein, has been described for a wide range of eukaryotic organisms including protozoa, yeasts, plants, nematodes and mammals. So far, the primary biological function of TCTP is still unclear, we speculated that TCTP may belong to a heat stable calcium binding protein (Kim et al., 2000), with capability of binding tubulin (Gachet et al., 1999), induced intracellular signaling (MacDonald et al., 1995), and be related with cell proliferation (Böhm et al.,

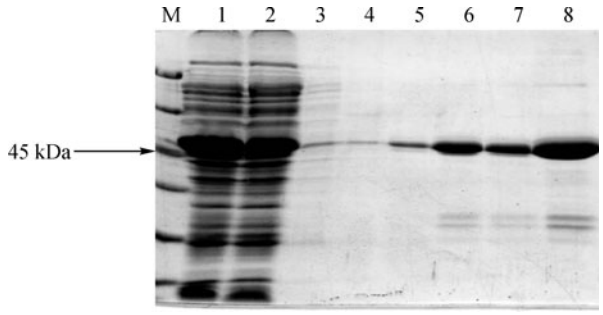

Figure 5 Purify of TaTCTP fusion protein. 1–2 are bacterial lysis supernatant; 3–5 are wash buffer; 6–8 are elution buffer, respectively.

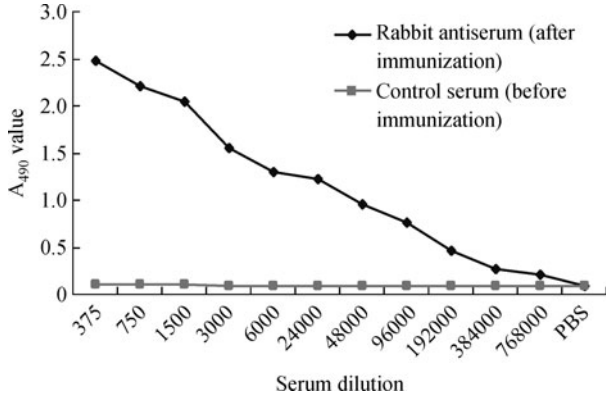

Figure 6 ELISA detection of antiserum titer.

1989). In animals, TCTP was related with the immune system of animal, and considered to play a role in the downstream transcriptional regulation (Xu et al., 1999). TCTP or its analog was also found in plants, but the research reports on its function were rare. Cao et al. (2006) found that *TCTP* gene only expressed in the plant root and stem, the expression of *TCTP* was induced by high temperature and salt, suggesting that it may be associated with the resistance of plants. Lopez and Franco (2006) showed that *TCTP* was constitutively expressed in the nutrition tissues of strawberry, and its expression was increased during fruit development, which may be involved in the regulation of fruit ripening. In addition, recent studies have shown that TCTP can prevent hydrogen peroxide-induced cell death (Nagano-Ito et al., 2009), promote the GDP-GTP exchange of Rheb (Dong et al., 2009), is a novel heat shock protein with chaperone-like activity (Gnanasekar et al., 2009).

Recent studies of plant TCTP often depend on the specific antibodies by Western blotting analysis. To facilitate the expression and purification of fusion proteins, in this study, the fragment was cloned into PET30a-GST vector with a GST-tag promoting the expression of fusion protein in the front of the insert, and His-tag by using genetic recombination in favor of purified fusion protein in the back-end of the insert. In this work, we successfully constructed the prokaryotic expression vector PET30a-GST-*TaTCTP*-His.

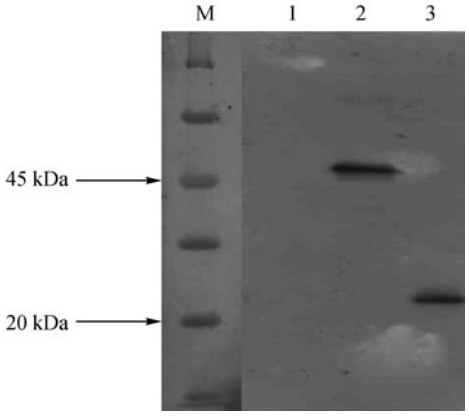

Figure 7 Western blotting verification of TaTCTP protein. M is marker proteins with molecular masses in kilodaltons. 1–3 represent BSA, the purified recombinant protein TaTCTP, and wheat whole protein. M is from SDS-PAGE, Lanes 1–3 are from western bloting.

TaTCTP fusion protein was expressed in *E. coli* Rosetta (DE3), and clear bands were obtained by SDS-PAGE separation and stained with Coomassie blue R250 (Fig.4). We explored the optimal conditions for the prokaryotic expression to obtain the best expression of fusion protein. Figure 4 showed that the fusion protein was highly expressed in the supernatant at low temperature, low IPTG concentration, so we took the conditions of 28°C, 0.1 mmol/L IPTG as the best conditions to carry out large-scale expression and purify fusion protein. In this experiment, we purified fusion protein by Ni-NTA affinity chromatography and obtained fusion protein with one clear main band by SDS-PAGE analysis. The titer and specificity of antibody was detected by indirect ELISA and Western blot, respectively. The titer of TaTCTP antiserum was 1:768000 detected by indirect ELISA. Western blot analysis showed that antibody can be bound not only specifically with TaTCTP fusion protein of Prokaryotic expression, but also with wheat whole protein. It suggested that the specificity of antibody is better, which can be used for subsequent functional studies.

In this paper, we successfully constructed the prokaryotic expression vector pET30a-GST-*TCTP*-His and purified the fusion protein by Ni-NTA affinity chromatography. The purified fusion protein was used as antigen to get the rabbit anti-TaTCTP antiserum. The results of Western blot indicated that the antiserum with the fusion protein and wheat whole protein can be specifically reacted with each other. Although we have a certain understanding about *TCTP*, the studies in plants are seldom. So the preparation of TaTCTP antiserum is of great significance on the research of biological function and complex regulatory networks in plants.

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