

Characterization of the Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) Gene from the Halotolerant Alga *Dunaliella salina* and Inhibition of Its Expression by RNAi

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Abstract Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) displays important functional diversity in mammalian and plants. So far, however, studies on GAPDH have not included the halotolerant, unicellular green alga *Dunaliella salina*. In the present study, a GAPDH cDNA was cloned and sequenced from *D. salina*. It was 1394 bp long, with an open reading frame of 1128 bp encoding 376 amino acid residues, and shared a high homology with other organisms. The coding region of the gene was heterologously expressed in *E. coli*, confirming that the gene cloned from *D. salina* is indeed GAPDH. Furthermore, the recombinant plasmid p7NBTFIR was constructed to express hairpin RNA (hpRNA) containing sequences homologous to the GAPDH gene to investigate the expression profile of GAPDH by RNAi in *D. salina*. The results of real-time quantitative PCR revealed that the relative transcription levels of the GAPDH gene in transformants G1 and G2 were reduced to 41.2% and 67.4%, respectively, of the wild-type *D. salina*. Observations under phase-contrast microscopy showed that the motility of the transformants cell was sluggish. The results of a photoaccumulation experiment showed that the cell motility of transformants G1 and G2 was less active than that of wild-type *D. salina*. The findings of this study may be useful for further studies on the subcellular localization and functional analysis of the GAPDH gene in microalgae.

Introduction

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is considered a classical glycolytic protein for its pivotal role in energy production. It is utilized as a model for protein and enzyme analysis and also as an internal control factor for relative quantitation of gene expression. More and more evidence suggests that GAPDH displays a number of diverse activities such as apoptosis and microtubule assembly [6, 12]. A previous study has demonstrated that the presence of glycolytic enzymes in flagella/cilia is likely to be widespread [3]. For example, glyceraldehyde-3-phosphate dehydrogenase-s (GAPDS) is a component of the mammalian sperm flagellum and essential for sperm motility and fertilization [7]. Compared with the comprehensive understanding of mammalian GAPDH, the functional characterization of algal GAPDH is still at an early stage. The study by Pazour et al. [9] showed that the flagellar proteome of *Chlamydomonas reinhardtii* contained all enzymes (especially GAPDH) of the late glycolytic pathway, suggesting that there is a mechanism to exclude soluble cytoplasmic proteins from the flagellum, and that the glycolytic enzymes are true flagellar components. *Dunaliella salina* is a halotolerant, unicellular, wall-less biflagellate alga [8]. Its resistance to high salinity, the simplicity of its cytoarchitecture, and its facility for cultivation make *Dunaliella* an important model organism to investigate fundamental molecular mechanisms [4, 14, 17]. A proteomic study of *D. salina* flagella using a shotgun strategy in our laboratory showed that peptides derived from GAPDH were present in the flagellar proteome, suggesting that this enzyme may be anchored to the flagellum of *D. salina*. Further research to characterize the functions of GAPDH in growth and development will

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reveal more detailed information on how GAPDH is regulated and how it may function and interact in *D. salina* flagella.

Despite its importance, there is little information available on the GAPDH gene in *D. salina*. Therefore, in the present study, we cloned and characterized a complete GAPDH cDNA from *D. salina*. Furthermore, a plasmid p7NBTFIR containing sequences homologous to the GAPDH gene was constructed based on hairpin RNA (hpRNA)-mediated genetic interference and transformed into *D. salina* cells to investigate inhibition effect by RNAi on expression profile of the GAPDH gene.

Materials and Methods

Algal Strain and Culture Conditions

D. salina UTEX-LB-1644 was purchased from the Culture Collection of Algae at the University of Texas, Austin. Cells of *D. salina* were grown in a modified medium at 26°C [1, 17].

Cloning of a GAPDH cDNA Fragment from *D. salina*

First, a pair of degenerate primers (5' GGNGGNGTNA(A/G)CA(A/G)GC 3' and 5' TANCCCCA(C/T)TC(G/A)TT(G/A)TC(G/A)TACC 3') was designed and synthesized corresponding to the conserved amino acid residues "GGVKQA" and "WYDNEWGY" of GAPDH. RT-PCR products were purified and cloned into *E. coli* JM 109 using the pMD18-T vector (TaKaRa), then sequenced. Homologue analysis of the sequence was performed using a blast search of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

Determination of the Transcription Start Site (TSS) and Terminator of the *D. salina* GAPDH Gene

According to the cloned fragment above, primers (5' GAA CACGCTGTGCCCTCAATCA 3' and 5' GCCTTCAC ATCGGCGGAGA AACTTGC 3') were designed and synthesized to amplify the 5'-ends of the GAPDH gene using the 5'-Full RACE Core Set (TaKaRa) to determine the TSS. A pair of primers (5' TGCGATGGCTACAAGCA CGACT 3' and 5' CAACTGCTTGGCTCCCT TCGTC 3') was used to amplify the 3'-end and the terminator of the GAPDH gene using the FirstChoice RLM-RACE Kit (Ambion). PCR was carried out with genomic DNA of *D. salina* as template and the amplified products recovered from 1% agarose gels were cloned into *E. coli* JM 109 and sequenced.

Characterization of the *D. salina* GAPDH Gene

The full-length cDNA of GAPDH was amplified using primers 5' CGCGGATCCATGGCTACAT CAATGGCGA AG 3' and 5' CGAGCTCTTAGGCGGCCACCTCTGG 3'. PCR products were purified, cloned, and sequenced as described above. BLAST and multiple sequence alignments were carried out at the NCBI Web site. The molecular weight and theoretical isoelectric point (pI) were also predicted at the Web site (<http://au.expasy.org/tools/protparam.html>). To investigate its heterologous expression, the coding sequence of *D. salina* GAPDH cDNA was subcloned into the prokaryotic expression vector pET28a(+) (Novagen) to produce a recombinant GAPDH fused to a His-tag as a fusion protein.

Construction of RNAi Vector p7NBTFIR

Eukaryotic expression vector p7NBT containing the bi-aphos-resistant (*bar*) gene was delivered into *D. salina* cells and transformants were confirmed by PCR and Southern blots [4]. Therefore, p7NBT was adopted to construct RNAi vector p7NBTFIR, which expressed hpRNA or self-complementary-containing sequences homologous to the GAPDH gene driven by the nitrate reductase (NR) promoter (Fig. 1). According to siRNA Target Finder at the Web site (http://www.ambion.com/techlib/misc/siRNA_finder.html), a 474-bp fragment, from bp 244 to bp 1112 of the GAPDH gene in *D. salina* was selected as the target sequence [2].

Nuclear Transformation and Screening of Transformants

Plasmid p7NBTFIR was transformed into *D. salina* by electroporation and the transformants were screened with phosphothricin (PPT) [4]. Transformations with plasmid p7NBT or with no plasmid were used as negative controls. PCR for detecting the integration of the *bar* gene in the transformant was performed using primers 5' CCCGGGAT CCATGAGCCCAGAACGACGC 3' and 5' CAATGAGC TCTCATCAGATCACGGTGAC 3', designed according to the sequence of the *bar* gene (GenBank X17220).

Analysis of Expression of the *D. salina* GAPDH Gene by RNAi

Real-time quantitative PCR was used to analyze inhibition of the GAPDH expression profile by RNAi in *D. salina*. Wild-type cells of *D. salina* served as negative controls. A pair of primers, 5' CAAGTTCTCCGCGATGTGA 3' and 5' GAA CACGCTGTGCCCTCAA 3', was used to amplify the GAPDH gene fragment (148 bp). The relative abundance of the β -actin gene was also determined and used as the internal

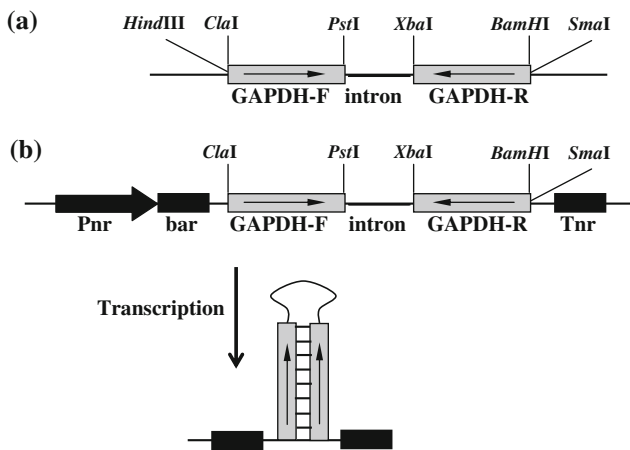


Fig. 1 Structural schematic of the recombinant expression vector p7NBT FIR used to induce RNAi. **a** Intermediate vector pUC19FIR. A 474-bp fragment, corresponding to the GAPDH coding sequence, was cloned in the forward (GAPDH-F) and reverse (GAPDH-R) orientations flanking a GAPDH intron/DNA spacer. **b** RNAi plasmid p7NBT FIR. A fusion fragment (GAPDH-F-intron-GAPDH-R, FIR) obtained from plasmid pUC19FIR by double digestion with *ClaI* and *SmaI* was cloned into the *ClaI-SmaI* sites of p7NBT in the 3'UTR of the bialaphos resistance (*bar*) gene. The transgene is under control of the *D. salina* nitrate reductase promoter (Pnr) and terminator (Tnr) and is designed to generate, upon transcription, an RNA containing a double-stranded stem-loop structure in the 3'UTR

control. The primers for amplifying β -actin gene fragment (194 bp) were 5' CCATCACCATCGGCAACG 3' and 5' GTCCGGCAATACCAGGGAACA 3'.

Transcription levels of the *D. salina* GAPDH gene were analyzed by real-time quantitative PCR with the 7300 Real Time PCR System (Applied Biosystems) using the SYBR Premix Ex Taq Kit (TaKaRa). Thermal cycling conditions were as follows: an initial heating cycle of 95°C for 10 min and 40 cycles of 94°C for 30 s, 52°C for 30 s, 72°C for 30 s, and 83°C for 5 s. The specificity of PCR amplification was checked by a melting curve program and electrophoresis on 1% agarose gels. All reactions were repeated independently three times. Data analysis was carried out using the comparative C_T ($2^{-\Delta\Delta C_T}$) method [5].

Photoaccumulation Experiments

Photoaccumulation experiments were performed according to the report by Vismara et al. [14].

Results

Determination of the TSS and the Terminator of the *D. salina* GAPDH Gene

Agarose gel analysis revealed that amplification with the degenerate primers resulted in a specific band of about

900 bp. BLAST-n analysis indicated that it had wide similarity to the known GAPDH genes, implying that it may be a partial fragment of the GAPDH gene of *D. salina*. Sequence analysis of the PCR product of 5'-RACE and 3'-RACE showed that the TSS (marked "G" in a box in Fig. 2) of the *D. salina* GAPDH gene is located 30 bp upstream of the initiator codon ATG, and that the 3'-untranslated region (UTR) possessed the typical low G+C content (45.1%) but not the typical polyadenylation signal TGTA described first in algae [11]. As shown in Fig. 2, a similar sequence (TGCAA), however, is present at positions 1223 to 1228 upstream from the poly(A) tail. Based on the overlaps among the cloned sequences, a full-length GAPDH cDNA of 1394 bp (poly[dA] not included) was spliced.

Characterization of the *D. salina* GAPDH Gene

The cloned GAPDH cDNA possessed an 1128-bp open reading frame (ORF) from bp 31 to bp 1158 of the sequence, except for a 30-bp 5' UTR and a 266-bp 3' UTR (Fig. 2). The nucleotide sequence data reported here were deposited in GenBank under accession no. EU447774. The predicted GAPDH protein was 376 amino acid residues in length, with a theoretical molecular weight of 40.27 kDa and a pI value of 9.14. According to NCBI protein-protein BLAST, it shared high identities with the following species: 94% with *D. viridis*, 83% with *Scenedesmus vacuolatus*, 81% with *Chlorella* sp. JP-2005, 80% with *C. incerta*, 79% with *C. reinhardtii*, 77% with *Chlorella* sp. W80, and 74% with *Ostreococcus tauri*. The findings indicate that the cloned sequence is a GAPDH cDNA from *D. salina*. Motif analysis revealed that GAPDH of *D. salina* shared the highly conserved GAPDH active site (ASCTTNCL) from 190 to 197 amino acid residues with the known GAPDHs [10].

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) showed that the culture sample induced by isopropyl- β -D-1-thiogalactopyranoside (IPTG) revealed high-level expression of recombinant GAPDH protein. Fusion proteins were <43.0 kDa, i.e., in agreement with the expected molecular mass of GAPDH. The results of biochemical assays demonstrated that the cDNA cloned in this study was a GAPDH gene derived from *D. salina*.

Screening of PPT-Resistant Transformants

Three transformants were shown to have PPT resistance through repeated PPT selection cycles. The two PPT-resistant transformants with plasmid p7NBT FIR were named G1 and G2, respectively, while one PPT-resistant transformant with plasmid p7NBT was named G3. The expected PCR fragments of the *bar* gene (about 560 bp)

Fig. 2 Full-length cDNA sequences (GenBank accession no. EU447774) of *D. salina* GAPDH and the deduced amino acid residues. The ORF of the 1128-bp cDNA extends from nucleotide 31 to nucleotide 1158, encoding a polypeptide of 376 amino acids. The putative initiation codon (ATG) and the stop codon (TAA) are boxed. The GAPDH active site (ASCTTNCL) is also boxed. The 5'-RACE inner primer and the gene-specific inner primer are underlined. In comparison with known sequences, the transcription start site (TSS; "G" in a box) of the *D. salina* GAPDH gene is located 30 bp upstream of the start codon ATG. The putative polyadenylation signal (TGCAA) is underlined

CGCGGATCCACAGCCTACTGATGATCAGTCGATG

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1   GAAAGCTTTAATTTAGCTCGGCCAGCGAATATGCTACATCAATGGCGAAGACCCGCTTC
61  ACGGGCAACATGGGTGCCCTGAAGAACGTACAGCGGTTGCTCCCGCCAGGGGCGCCGTG
121 T G N M G A L K N V Q R V A P A R G A V
    AAGATGGAGGTCGTGGCCCAGAAGAAGCTGCGCGTGGCCATCAACGGCTTTGGCCGTATT
181 K M E V V A Q K K L R V A I N G F G R I
    GGCCGCAACTTCTGCGCTGCTGGGAGGGCAGGCAGGACTCCCTGCTGGACGTCGTGTGC
241 G R N F L R C W E G R Q D S L L D V V C
    GTGAACGACTCCGGTGGTGTGAAGCAGGCCTCTCACCTGCTTAAGTATGACACCACCTG
301 V N D S G G V K Q A S H L L K Y D T T L
    GGCAAGTTCTCCGCCGATGTGAAGCCGTCGACGAGAAGACTCTCAGCGTGAACGGCAAG
361 G K F S A D V K A V D E K T L S V N G K
    AACATTGCCGTGGTGTCCAGCCGCGATCCCCTCAGCTGCCCTGGAAGCCATGAACATC
421 N I A V V S S R D P T T Q L P W K A M N I
    GACCTGGTGATTGAGGGCAGCGGTGTTTCATTGACACCCTGGCGCTGGCAAGCATC
481 D L V I E G T G V F I D T P G A G K H I
    CAGGCCGGCCGAAGAAGGTCCTGATCACCGCCCCGCCAAGGGCTCTGACATCCCCACC
541 Q A G A K K V L I T A P A K G S D I P T
    TTTGTGGTCGGCGTGAAGTGGCTACAAGCAGACTACCCCATCGTCTCCAACGCC
601 F V V G V N C D G Y K H D Y P I V S N A
    TCTTGCACCACCAACTCTGGCTCCCTTCGTCAAGGTGCTGCATGAGAAGTTCCGGTGTG
    S C T T N C L A P F V K V L H E K F G V
661 GTTAAGGGTACCATGACTACCACCCACTCTTACACTGGTGACCAGCGTCTGCTGGATGCC
721 V K G T M T T T H S Y T G D Q R L L D A
    TCCCACCGGATCTGCGCGTGCCCGTGCTGCTGCCCTGAACATTTGTGCCACCACCACT
781 S H R D L R R A R A A A L N I V P T T T
    GGTGCGGCCAAGGCTGTGGCTCTGGTGTGTCGAGAACTGAAGGGCAAGCTGAATGGTGTG
841 G A A K A V A L V L P E L K G K L N G V
    GCTCTGCGTGTGCCACCCCAACGTCTCCATCGTGGATCTGGTTGTCAGGTGGAGAAG
901 A L R V P T P N V S I V D L V V Q V E K
    AAGACCTTCGCTGAGGAGGTGAACAACGCCTTCCGCGAGGCTGCTGCTGGCCCCATGAAG
961 K T F A E E V N N A F R E A A A G P M K
    AATGTGCTGGCTGTGGCTGATGAGCCTCTGGTGTCTGCTGACTTCAAGGGCATGGACCAG
1021 N V L A V A D E P L V S A D F K G M D Q
    AGCACTGCCATCGACTCTGCCCTGACCATGGTCAATGGTATGACATGGTCAAGGTTGTT
1081 S T A I D S A L T M V M G D D M V K V V
    GCCTGGTACGACAACGAGTGGGGCTACAGCCAGCGTGTGTTGGACCTTGTGAGATCACC
1141 A W Y D N E W G Y S Q R V V D L A E I T
    CCCAGAGGTGGGCCGCCATAAGGCCATGATTTTGGCAAAGGCATGTGCTCCGGATACCTC
1201 A Q R W A A *
    TTCCATCACCACTGCTTGGCTTTAGGATTAGATCAGATTTCGGAACCTTCTGTCC TGCAAAG
1261 CCCTAAAACAGCACTCCTCCCAACATACTTCTCATGATTGGGTTGGGTCAATTAAGGTCT
1321 TTATAACTGCAGGCATCTAGATACCATAACCAGCATTTTACACGATCGTGTATCATCCC
1381 ACGCACAGATAATGACTACCTGCTGTGAAGCAGTGCCTTATACTAAAAAAAAAAAAAAAA
    
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were detected in all three transformants but not in wild-type *D. salina* cells. Sequencing of the PCR products indicated that the amplified DNA fragment was consistent with the coding region of the *bar* gene. These results indicated that both the vectors were transformed into *D. salina* cells.

GAPDH Expression Profile in *D. salina*

As the $2^{-\Delta\Delta CT}$ values reflected the relative transcripts of the target gene in the experimental group versus those in the negative control, the result suggested that the transcription level of GAPDH in the p7NBTFIR-treated group changed compared to that of the control. As shown in Fig. 3, the $2^{-\Delta\Delta CT}$ values of the transformant G1 and G2

were 0.4118- and 0.6736-fold, respectively, of the relative GAPDH transcript control. The $2^{-\Delta\Delta CT}$ value of the transformant G3 was 0.9659, indicating that GAPDH expression was nearly the same in both the p7NBT-treated group and wild-type cells. The results showed that the transcription levels of GAPDH in transformants G1 and G2 obviously decreased, suggesting that GAPDH expression could be inhibited strongly by transformation with p7NBTFIR in *D. salina*.

Photoaccumulation Experiments

As shown in Fig. 4, density profiles of wild-type and transformant G3 cells were similar, corresponding to the

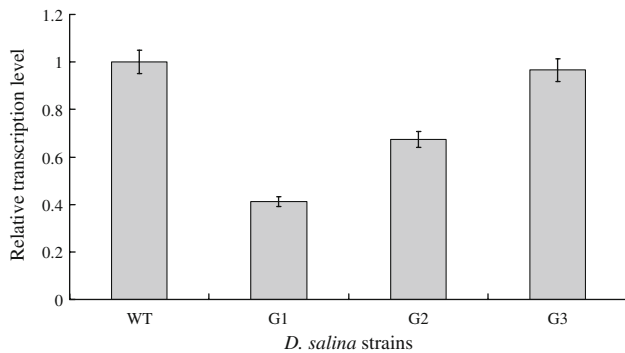


Fig. 3 Inhibition of GAPDH expression by RNAi in *D. salina*. Relative transcript levels of the GAPDH gene in different transformants were analyzed by real-time PCR. Error bars show standard deviations for three independent experiments. WT—wild-type cells of *D. salina*, which served as negative control; G1 and G2—two PPT-resistant transformants treated with plasmid p7NBTFIR; G3—PPT-resistant transformant with plasmid p7NBT

accumulated cells in the illuminated spot of the dish. However, the density profiles of transformants G1 and G2 were much lower than those of wild-type and transformant G3 cells.

Discussion

In this study, we cloned and characterized the gene encoding GAPDH of *D. salina*. Multiple sequence alignments revealed that the deduced amino acid sequence of the *D. salina* GAPDH gene shared high homology with that of many algal species and other organisms. The results suggest that the cloned sequence is a GAPDH cDNA from *D. salina*. Previous research by Pazour et al. [9] and our proteomic study of *D. salina* flagella indicate that GAPDH is likely anchored to the flagellum. The cloning and characterization of *D. salina* GAPDH in the present study will be useful for subcellular localization of GAPDH and for investigation of its function and interaction in microalgae.

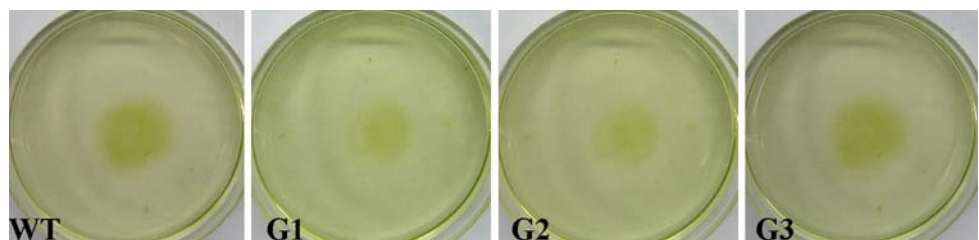
To improve the silencing efficiency of a target gene in *D. salina*, an integrated transformation system is required for stable gene expression, where an algal endogenous expression element or homologous integrating platform is needed. An RNAi construct containing sequence homologous to the phytoene desaturase gene (*pds*) driven by the

CaMV promoter induced transient suppression in *D. salina* [13]. In the present study, expression of the GAPDH gene was controlled by the NR promoter, an endogenous promoter from *D. salina*. Previous study revealed that the promoter of the NR gene of *D. salina* can be used for controllable expression of heterologous genes using the inducer nitrate and the repressor ammonium [4]. Therefore, cells of the transformant are normally cultured in medium containing ammonium as nitrogen source, and the transformant can be transferred to the medium containing nitrate to induce inhibition of the gene expression when it is needed. This attractive trait is useful for further developing RNAi methodology and for extending analysis of other intriguing genes in *Dunaliella*.

Gene silencing tends to achieve a high efficiency when sequences of more than 300 bp are used [15]. Hence 474 bp of the target fragment of GAPDH was adopted in this study to construct the hpRNA expression vector. At the same time, the efficiency of gene silencing using such sequences might be enhanced by fusing the sense and reverse sequences with a “spacer” in the hpRNA constructs [15, 16]. Therefore, in our construction, a 259-bp sequence cloned from one of the GAPDH introns was inserted into the two self-complementary regions. The relative transcription level of GAPDH in p7NBTFIR-treated cells versus nontreated cells was quantitated and evaluated via real-time PCR. Transcription of GAPDH in transformants G1 and G2 was reduced to about 41.2% and 67.4%, respectively, of the control cells. The cell motility of transformants G1 and G2 under phase-contrast microscopy is sluggish compared with the vigorous progressive motility of wild-type *D. salina* (data not shown). Furthermore, the results of the photoaccumulation experiment showed that the cell motility of transformants G1 and G2 was less active than that of wild-type *D. salina*, suggesting that GAPDH plays an important role in the motility of *D. salina* flagella. The findings of this study may be helpful for further studies of subcellular localization and functional analysis of the GAPDH gene in microalgae.

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Fig. 4 Results of photoaccumulation experiments. Photoaccumulation of *D. salina* wild-type (WT) cells and transformants G1, G2, and G3 after 3 h are shown in four petri dishes



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