

Full-length cloning and characterization of abiotic stress responsive *CIPK31-like* gene from finger millet, a drought-tolerant crop

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Regulatory proteins such as protein kinases are known to play a crucial role in plant stress acclimation pathways. In this study, we report the identification and characterization of an abiotic stress responsive protein kinase called CBL Interacting Protein Kinase (*EcCIPK31-like*) from finger millet, a drought-tolerant crop. PCR-based approach was followed to clone 1350 bp coding region that encodes a 449 amino acid protein with a calculated molecular mass of 50.69 kDa. The conserved domain analysis revealed the presence of CIPK domain with 90% sequence similarity with fox tail millet *SiCIPK31-like* gene. We also report its upregulation under salinity, desiccation, oxidative and temperature stresses at seedling level in finger millet. Drought stress at whole-plant level significantly induced the expression of *EcCIPK31-like*, indicating that the gene is linked to drought signalling pathways. The stress-responsive nature of *EcCIPK31-like* to diverse stresses indicates that the gene could regulate multiple cellular tolerance traits and its further functional validation can highlight the relevance in abiotic stress acclimation in plants.

Keywords: Abiotic stress signalling, finger millet, full-length cloning, protein kinase.

PLANTS respond to various stimuli by activating various molecular, cellular and physiological processes, involving several signalling cascades required for developmental and adaptive events. The timely response to any external stimuli is governed by efficient signal perception and the downstream signal transduction. The signal is conveyed to the nucleus by an array of secondary messengers like inositol 1,4,5-trisphosphate (IP₃), diacylglycerol (DAG) and calcium (Ca²⁺) which are generated by phospholipid signalling systems¹. The different abiotic stresses such as drought, salinity, high and low temperature, etc. increase the Ca²⁺ concentration in cell cytoplasm, which acts as a major secondary messenger to

transduce the extracellular stimuli². The cellular Ca²⁺ signals are detected and further transmitted by sensor molecules. The three main classes of Ca²⁺ sensors identified in plants are calmodulin (CaM) and CaM-related proteins, Ca²⁺-dependent protein kinases (CDPKs) and calcineurin B-like proteins (CBLs). In higher plants, CBLs represent a distinct group of Ca²⁺ sensors which play a key role in decoding oscillating Ca²⁺ signatures by specifically interacting with and also regulating a family of protein kinases called CBL interacting protein kinases (CIPKs)³. Plants have specific CIPKs which are Ser/Thr protein kinases that interact with CBLs containing four helix-loop-helix domains for Ca²⁺-binding⁴. Activated CIPKs further transduce the Ca²⁺ signals by phosphorylating downstream signalling components⁵. In *Arabidopsis thaliana*, *Oryza sativa* and *Populus* species, 26, 30 and 25 CIPK genes have been reported respectively⁶⁻⁸. Although there are many reports on the role of CIPKs in imparting abiotic stress tolerance⁹⁻¹¹, there could be vast diversity in the functions exhibited by different CIPKs. This could be attributed to the superior nucleotide/polypeptide assembly existing in different CIPKs, making them functionally more efficient^{12,13}. In this regard, identification and analysis of gene function from stress-adapted crops assumes significance. Stress-tolerant crops can exhibit superior tolerance traits by adjustments at genome, transcriptome or proteome level to deliver a stable functional protein, relevant for the tolerance mechanism/trait. Finger millet (*Eleusine coracana* (L.) Gaertn.), a drought-tolerant monocot crop, can serve as an ideal system to prospect superior genes associated with tolerance^{14,15}. In the present study, we identify and validate a novel drought-responsive CIPK from finger millet.

Representation of *CIPK31-like* gene was observed in the finger millet drought-specific cDNA library developed in a previous study¹⁵. Hence, we cloned *EcCIPK31-like* from finger millet exposed to drought stress. To create drought stress, one-month-old finger millet (variety GPU-28) plants were subjected to 50% soil field capacity (FC) by gravimetric approach¹⁶. The stressed leaf tissue was used to extract total RNA and the first strand cDNA was used to clone *EcCIPK31-like* gene. Since the finger millet genome is not sequenced to clone the gene, CIPK-like sequences from different monocot species were collected from the NCBI database (<http://www.ncbi.nlm.nih.gov/>) and analysed to design primers (*EcCIPK31-F1* and *EcCIPK31-R1*) (Table 1). The 3' and 5' ends of the gene were cloned by RNA ligase-mediated rapid amplification of cDNA ends (RACE) approach using GeneRacer Kit (InvitrogenTM, California, USA) following the manufacturer's instructions. For 3' and 5' RACE, PCR was performed with *EcCIPK31-F2* (Table 1) and GeneRacer 3' primers, and *EcCIPK31-R2* (Table 1) and GeneRacer 5' primers respectively, by following the manufacturer's PCR conditions. The diluted primary PCR product (ten fold) was used as template in the secondary

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Table 1. List of primers used in the study

Primers	Sequence 5'-3'	Application
<i>EcCIPK31-F1</i>	GGAAGGTTGAAGGAGGAGGAAGCAGC	Partial length amplification of <i>EcCIPK31-like</i> gene
<i>EcCIPK31-R1</i>	GAACTTTTGGAACCCAGAGTGTCCCC	
<i>EcCIPK31-F2</i>	CACGAACTGACAGGCACTGAATCTGG	3' RACE
<i>EcCIPK31-R2</i>	CAGCAGCTGCACCATCAATAAGCCTC	5' RACE
<i>EcCIPK31-R3</i>	GCTCCTGAGGTATCGAAGATGGAGG	5' RACE-nested PCR
<i>EcCIPK31-F3</i>	CGATGTACAAGGCTAAAAGAGCTGC	Full-length amplification of <i>EcCIPK31-like</i> gene
<i>EcCIPK31-R4</i>	GAAACTGGTTCATGCCGCTGCGC	
<i>EcCIPK31-RT-F5</i>	CTACCTGGATGAATGTAGACGCTG	Gene expression analysis
<i>EcCIPK31-RT-R4</i>	GTGATGATTTCCTTGGAGGAC	
<i>EcActin-F</i>	TCCATAATGAAGTGTGATGT	Internal control for gene expression
<i>EcActin-R</i>	GGACCTAGACTCGTCATACT	

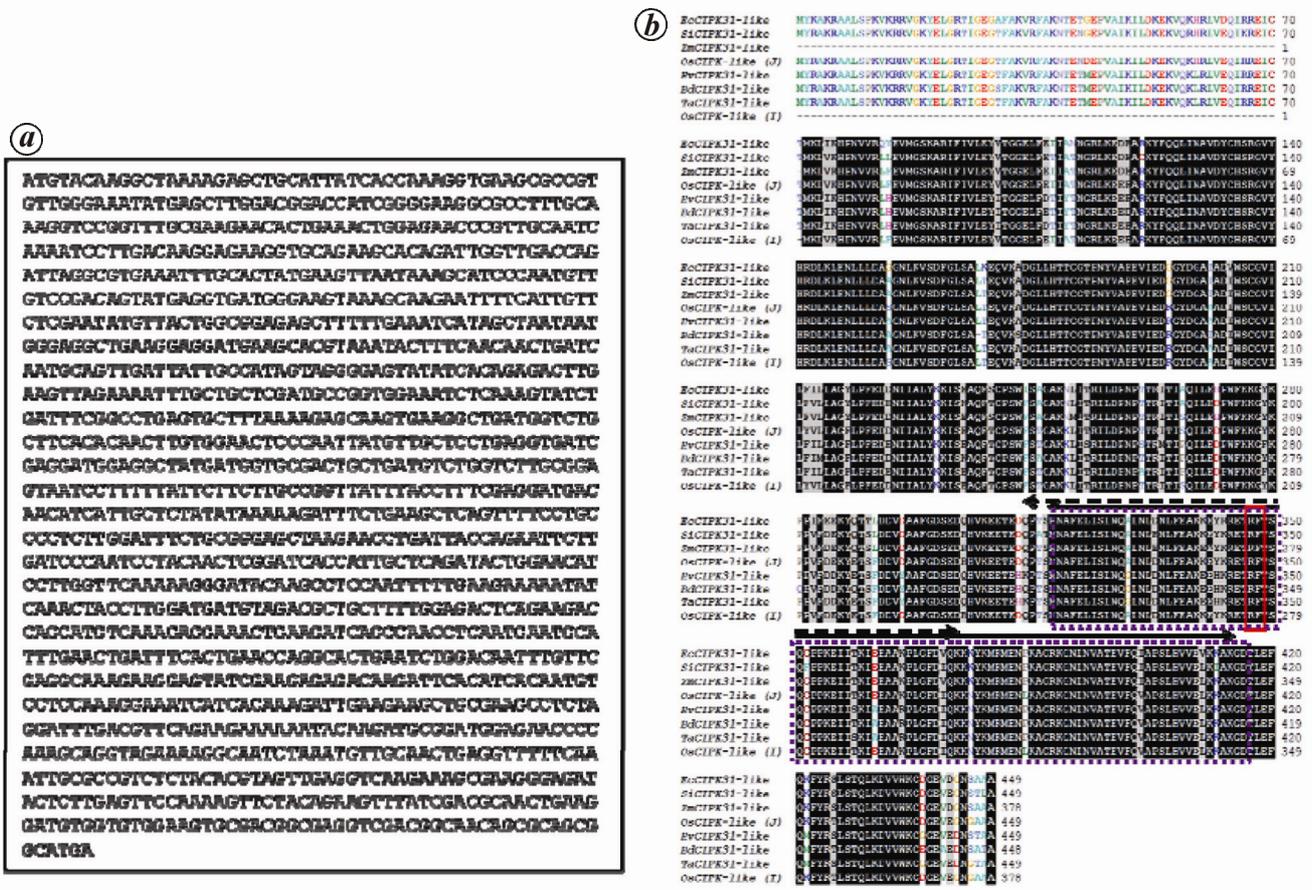


Figure 1. a, Full-length coding sequence of *EcCIPK31-like* gene. b, Alignment of *Eleusine coracana* CIPK31-like with related CIPK31-like proteins from *Setaria italica* CIPK31-like; *Zea mays* CIPK31-like; *Oryza sativa* (japonica) CIPK-like; *Hordeum vulgare* CIPK31-like; *Brachypodium distachyon* CIPK31-like; *Triticum aestivum* CIPK31-like; *Oryza sativa* (indica) CIPK-like. The amino acid residues shaded with black and grey have 100% and 90% sequence similarity of *EcCIPK31-like* with respect to the other species. The CIPK domain amino acid residues are marked with a violet dotted box; black arrow line denotes the CBL interacting site, whereas black split arrow lines show the NAF domain. The putative PP2C binding site is highlighted in red box.

PCR with gene-specific *EcCIPK31-R3* (Table 1) and GeneRacer 5' nested primers. The PCR products were cloned (MBI Fermentas, Life Sciences, USA), and sequenced (ABI 3730X1 sequencer). The full-length CDS of *EcCIPK31-like* gene was amplified with *EcCIPK31-F3* and *EcCIPK31-R4* (Table 1) primers designed using the

sequence information generated from 3' and 5' RACE. Using cDNA as template, PCR was carried out in 50 µl reaction volume containing *TaKaRa* LA *Taq* DNA polymerase according to the manufacturer's instructions (TAKARA BIO INC, Japan). The PCR products were cloned and sequenced.

Full-length gene sequence was subjected to BLASTn analysis (<http://www.ncbi.nlm.nih.gov>). Sequence alignment was done with the Sequence Manipulation Suite software¹⁷ and phylogenetic analysis was performed based on the neighbour-joining (NJ) method with 1000 bootstrapping replicates using the program MEGA 4.1 (ref. 18).

The stress responsiveness of *EcCIPK31-like* was examined at seedling as well as whole-plant level. Finger millet seedlings were grown in petri plates under control condition for four days and subsequently subjected to rapid desiccation (air-dried), heat (42°C) and cold (4°C) stresses for 3 h. To induce salinity and oxidative stress, finger millet seedlings were exposed to NaCl (150 mM) and methyl viologen (10 µM) respectively, for 3 h. At the end of the stress period, shoot and root tissues were collected for total RNA isolation and subsequent cDNA synthesis. Drought stress was imposed on 20-day-old plants raised in battery pots by gravimetric approaches^{15,16}. Soil field capacities (FCs) of 80%, 60%, 40% and 20% were maintained to create drought stress by controlled irrigation^{15,16}. Eight days post-stress imposition, leaf tissues from both stressed and non-stressed plants were collected for expression analysis. The tissues harvested were flash frozen in liquid nitrogen and stored at -80°C, or directly used to extract total RNA. Total RNA was extracted from the flash frozen leaf tissues using the protocol described by Datta *et al.*¹⁹. Total RNA (5 µg) was reverse-transcribed into cDNA in a random primed reaction using Molony–Murine Leukaemia Virus Reverse Transcriptase (MMLV-RT; MBI Fermentas, Life Sciences, USA)²⁰ and used as the template. RT–PCR was carried out in 20 µl reaction containing template cDNA, 2 µl PCR buffer (10×), 2 µl dNTPs (2 mM dNTPs), forward and reverse primers (3 pmol µl⁻¹ each; *EcCIPK31-RT-F5* and *EcCIPK31-RT-R4*) (Table 1), and 1 U *Taq* DNA polymerase (Kapa Biosystems, South Africa). The housekeeping gene, *actin*, was used as internal control using gene-specific primers (*EcActin-F* and *EcActin-R*) (Table 1). The expression levels were represented as relative expression estimated as ratio of *EcCIPK31-like/EcActin*, and heat maps were developed for the expression profiles of genes using Image J 1.45s (<http://imagej.nih.gov/ij>) and MeV (Multi Experiment Viewer v4.4 software, www.tm4.org).

Initial PCR with cDNA as template yielded a partial length gene fragment of 930 bp, and homology search showed 90% similarity with the predicted sequence of *Setaria italica* CBL interacting protein kinase 31-like (XM_004984505.1). The 3' and 5' RACE approach generated an additional sequence of 151 and 463 bp respectively. Further, 1350 bp full-length coding sequence (CDS) was amplified and sequenced. BLASTn analysis showed 90% sequence similarity with the predicted sequence of *S. italica* CBL-interacting protein kinase 31-like followed by 88% similarity with the sequence of *Zea*

mays CIPK-like protein 31 (XM_008676540.1). Hence, the cloned full-length sequence was designated as *EcCIPK31-like* gene and deposited in NCBI GenBank under the accession number KT288194 (Figure 1 a). The translated amino acid prediction showed that the CDS encodes a 449 amino acid protein with a calculated molecular mass of 50.69 kDa and a theoretical pI of 8.43. The alignment of *EcCIPK31-like* gene with other related species showed the presence of CIPK conserved domain with CBL interaction site, NAF domain and PP2C binding site (Figure 1 b). The phylogenetic analysis showed that *EcCIPK31-like* was found to be more closely related to *S. italica* with 90% homology followed by maize and rice with 88% and 87% homology respectively (Figure 2).

The stress-responsive nature of *EcCIPK31-like* was revealed by targeted stress assays performed at seedling as well as whole-plant level. In the seedling system, maximum expression was noticed under salinity, oxidative, heat and cold stresses in shoot tissues (Figure 3 a and c). *EcCIPK31-like* showed higher expression in root tissue, 3 h after multiple stress treatments compared to control (Figure 3 b and c). Further, the expression pattern of the gene was examined in leaf tissues subjected to drought stress at whole-plant level (Figure 4 a). Higher expression of *EcCIPK31-like* was observed under drought stress conditions (at 20%, 40%, 60% and 80% FCs), when compared to well-irrigated (100% FC) condition (Figure 4 b). As shown in Figure 4 c, maximum expression of *EcCIPK31-like* was observed under 20% FC.

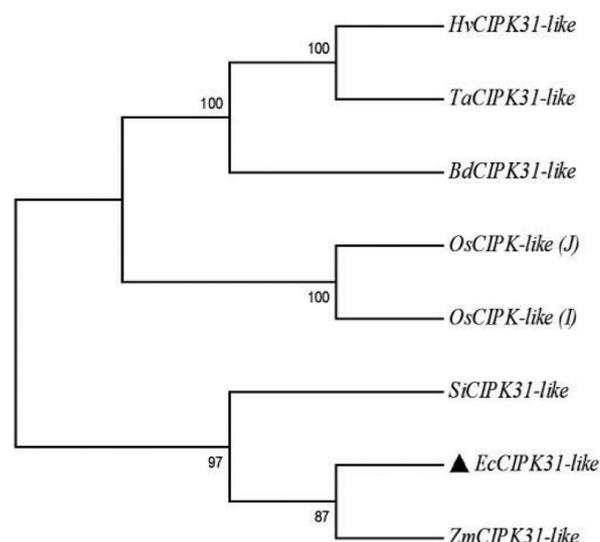


Figure 2. Phylogenetic analysis of nucleotide sequence of *EcCIPK31-like* with CIPK of other species. Sequences were aligned using CLUSTALX and the phylogenetic tree was constructed in MEGA 4.1 program. The *EcCIPK31-like* gene is shown in a full triangle. The appended CIPK31-like sequences are as follows: *S. italica* CIPK31-like (XM_004984505.1); *Z. mays* CIPK31-like (XM_008676540.1); *O. sativa* (*japonica*) CIPK-like (EU703794.1); *H. vulgare* CIPK31-like (JN655682.1); *B. distachyon* CIPK31-like (XM_003557984.2); *T. aestivum* CIPK31-like (JX625142.1); *O. sativa* (*indica*) CIPK-like (EF576522.1).

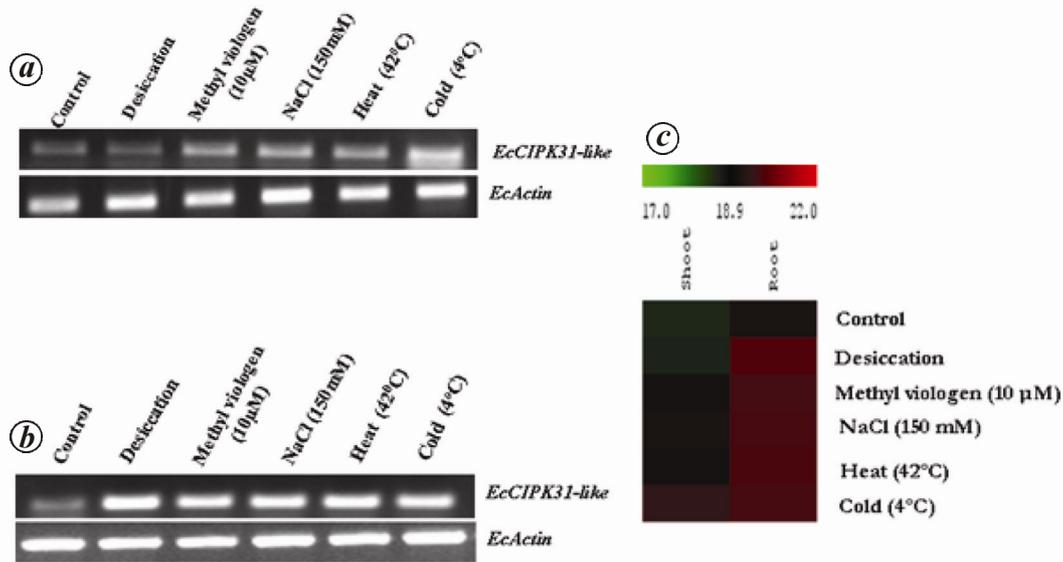


Figure 3. RT-PCR analysis of *EcCIPK31-like* in finger millet. *a*, *b*, Expression patterns in (*a*) shoot and (*b*) root tissues, subjected to different abiotic stresses [rapid desiccation, NaCl (150 mM), methyl viologen (10 μM), heat (42°C) and cold (4°C) for 3 h]. *c*, Heat map representing relative expression levels of *EcCIPK31-like* in finger millet shoot and root tissues subjected to different abiotic stresses.

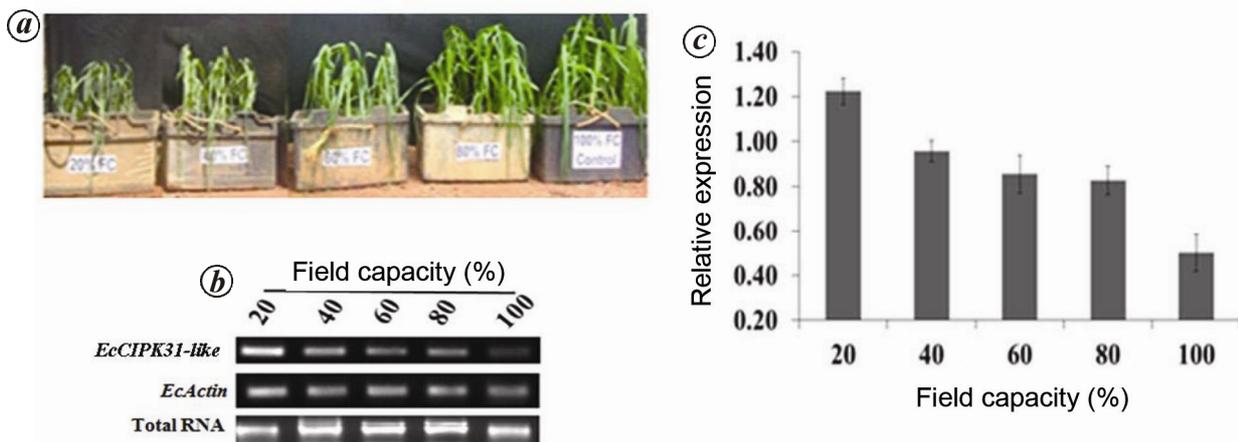


Figure 4. *a*, Phenotype of finger millet plants maintained under different levels of drought stress (soil field capacity, FC) imposed by gravimetric approach. *b*, Expression of *EcCIPK31-like* under control (100% FC) and drought stress (20%, 40%, 60%, 80% FC) conditions. *c*, Relative expression levels of *EcCIPK31-like* under different levels of drought stress. Data shown are mean \pm SE from three independent experiments.

The microarray expression profiling of different CBLs and CIPKs in rice revealed that *OsCIPK31* was highly expressed under cold, drought and salt stresses, and exhibited differential expression across developmental stages¹². This suggests that *CIPK31* has a regulatory role in plant development and abiotic stress responses. There are other CIPKs, for example, *CaCIPK6* which plays a role in plant development and salt stress tolerance as proven in transgenic tobacco plants ectopically expressing the gene⁹. Similarly, the expression of *AtCIPK6* was reported to be induced by ABA, osmotic and salt stresses, and the protein was found to interact with *AtCBL1/3/4* to confer salt tolerance⁹. In addition, the differentially

induced expression of *OsCIPK* genes by different abiotic stresses was proposed to be responsible for improved stress tolerance in rice¹⁰. It was also reported that the overexpression of halophytic gene, *NtCIPK2* in *Escherichia coli* resulted in better bacterial growth under high salinity and osmotic conditions, in addition to dehydration and extreme temperatures compared to control¹¹. More recently, it was reported that the overexpression of *GhCIPK6* significantly enhanced the tolerance to salt, drought and ABA stresses in transgenic *Arabidopsis*²¹. These results are in agreement with the expression pattern of *EcCIPK31-like* gene, which could be a novel regulatory protein, and its further validation could shed light on

the complex abiotic stress signalling networks. Since there are multiple CIPKs, they will have specific and overlapping roles in signalling, probably by interacting with diverse cellular proteins. The CBL–CIPK pathway is widely studied in rice and other crops, with 10 CBLs and 30 CIPKs¹² reported in rice, among which *OsCIPK31* is a more recently identified member involved in germination and seedling growth under abiotic stress conditions²². *OsCIPK31* was reported to have the maximum number of eight splice variants among all the *OsCIPKs*, which reveals its probable functional significance¹². The functional relevance of these splice variants is yet to be characterized. In drought-tolerant crops like finger millet, the alternative splicing phenomenon can have more relevance in adjusting the system to stress challenges and can thus harbour novel stress regulatory CIPKs. The availability of nuclear genome of finger millet in future might unravel complex regulatory networks associated with CIPKs. Elucidating the stress responsiveness of *EcCIPK31-like* is an important step towards understanding its functional significance and identifying key interacting partners. These results show that *CIPK-like* protein from finger millet could be linked to the drought hardiness of the crop; however, the molecular mechanisms need to be examined.

Thus the present study describes the cloning of *EcCIPK31-like* from finger millet. The stress responsiveness of *EcCIPK31-like* indicates that it is an active component of diverse stress signalling pathways which may be essential for imparting tolerance to multiple stresses in finger millet. Functional validation by overexpression or down-regulation approaches can provide insight into the relevance of the gene under stress, which needs to be examined.

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