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Requirement of CHROMOMETHYLASE3 for somatic inheritance of the spontaneous tomato epimutation Colourless non-ripening

Weiwei Chen1*, Junhua Kong1*, Cheng Qin1*, Sheng Yu2*, Jinfan Tan1, Yun-ru Chen2, Chaoqun Wu1, Hui Wang1, Yan Shi2, Chunyang Li3, Bin Li1, Pengcheng Zhang1, Ying Wang1, Tongfei Lai1, Zhiming Yu1, Xian Zhang1, Nongnong Shi1, Huizhong Wang1, Toba Osman3, Yule Liu4, Kenneth Manning3, Stephen Jackson3, Dominique Rolin5, Silin Zhong2, Graham B. Seymour6, Philippe Gallusci5 & Yiguo Hong1,3

1Research Centre for Plant RNA Signalling, College of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 310036, China, 2State Key Laboratory of Agrobiotechnology, School of Life Sciences, The Chinese University of Hong Kong, Hong Kong, 3School of Life Sciences, University of Warwick, Coventry CV4 7AL, UK, 4MOE Key Laboratory of Bioinformatics, Centre for Plant Biology, School of Life Sciences, Tsinghua University, Beijing 100084, China, 5UMR Fruit Biology and Pathology, Bordeaux University, INRA, Villenave d’Ornon 33883, France, 6Plant and Crop Science Division, School of Biosciences, University of Nottingham, Loughborough, Leics LE12 5RD, UK.

Naturally-occurring epimutants are rare and have mainly been described in plants. However how these mutants maintain their epigenetic marks and how they are inherited remain unknown. Here we report that CHROMOMETHYLASE3 (SlCMT3) and other methyltransferases are required for maintenance of a spontaneous epimutation and its cognate Colourless non-ripening (Cnr) phenotype in tomato. We screened a series of DNA methylation-related genes that could rescue the hypermethylated Cnr mutant. Silencing of the developmentally-regulated SlCMT3 gene results in increased expression of LeSPL-CNR, the gene encodes the SBP-box transcription factor residing at the Cnr locus and triggers Cnr fruits to ripen normally. Expression of other key ripening-genes was also up-regulated. Targeted and whole-genome bisulfite sequencing showed that the induced ripening of Cnr fruits is associated with reduction of methylation at CHG sites in a 286-bp region of the LeSPL-CNR promoter, and a decrease of DNA methylation in differentially-methylated regions associated with the LeMADS-RIN binding sites. Our results indicate that there is likely a concerted effect of different methyltransferases at the Cnr locus and the plant-specific SlCMT3 is essential for sustaining Cnr epi-allele. Maintenance of DNA methylation dynamics is critical for the somatic stability of Cnr epimutation and for the inheritance of tomato non-ripening phenotype.

Spontaneous epimutations can result from heritable changes in DNA methylation without alteration in the underlying sequence, but these changes can influence gene expression and associated phenotypes1-5. Indeed epimutations can affect inbred traits in plants and animals6-14. However natural epigenetic variations are rare and little is known about how spontaneous epimutations retain their heritable stability1-5. In plants, methylation occurs at cytosines in CG, CHG and CHH contexts (where H = A, T, C) through the combined enzymatic activity of DOMAINS REARRANGED METHYLTRANSFERASEs (DRMs), METHYLTRANSFERASE1 (MET1) and the plant specific CHROMOMETHYLASEs (CMTs)15,16. These enzymes are required for RNA-directed DNA methylation (RdDM) and methylation maintenance. In Arabidopsis, DRM2 catalyses de novo methylation in all sequence contexts and CMT2 is involved in non-symmetrical methylation while MET1, CMT3 and DRM2 participate in methylation maintenance at the CG, CHG and CHH sites, respectively15,16.

The tomato Colourless non-ripening (Cnr) is one of the best characterized naturally occurring epimutants1. Cnr differs from structural epi-variants such as CmWIP, FWA, FOLT1 and SP1117-20 in Arabidopsis, melon and Brassica, of which the epigenetic changes are either induced by transposon or trans-acting small RNAs, or genetic non-ripening mutants such as tomato rin, ripening-inhibitor21. Cnr contains eighteen hypermethylated cytosines
in a 286-bp region of the LeSPL-CNR promoter at the Cnr locus and the Cnr epimutation and phenotype are very stable. We only observed four Cnr fruits with revertant sectors showing red stripes out of thousands of fruits grown over more than twenty years. In this paper, using the spontaneous Cnr epimutant together with VIGS-based gene functional screening, targeted and whole-genome DNA methylation profiling and qRT-PCR assay, we investigate the mechanism responsible for somatic inheritance of Cnr. We unravel that SlCMT3 silencing results in reduction of DNA methylation and leads to Cnr-to-ripening reversion in tomato. Our results demonstrate that SlCMT3, possibly along with other key components including SlCMT2, SlDRM7 and SlMET1 in the RdDM and methylation maintenance pathways, is required to maintain the Cnr epi-allele, and CMT3 possesses an important role in epigenetic regulation of structural genes such as transcription factors in addition to its role in maintaining the methylation of repetitive DNA and transposon-related sequences.

**Results**

Silencing of DNA methylation-associated genes affects Cnr fruit ripening. Cnr phenotype could be recreated in normal fruits by repression of LeSPL-CNR or by increasing methylation level in the 286-bp region (Supplementary Fig. 1), demonstrating that hypermethylation causes the phenotype. The eighteen hypermethylated cytosines in a 286-bp region of the LeSPL-CNR promoter are thought to be responsible for the non-ripening phenotype (Fig. 1a). To uncover the mechanism guarding the stability of the Cnr epi-allele, we used Potato virus X (PVX)-based VIGS to silence a range of DNA methylation-associated genes including SlDRM7, SlMET1, SlCMT2, SlCMT3 and SlCMT4 (Fig. 1b). These genes were selected based on sequence homology to the well-characterized Arabidopsis DNA-methyltransferases (DMTs; Supplementary Fig. 3). Specific cDNA fragments corresponding to each of the SlDMT genes were cloned into the PVX-based VIGS vector (Fig. 1b). It is worthwhile noting that nucleotide similarities among sequences of VIGS inducers are mostly around 30% or lower (Supplementary Table 1). Considering the requirement of perfect complementarity between silencing inducer and target sequences for small RNA (siRNA and microRNA)-mediated silencing in plants, we expect that these constructs including PVX/SlCMT2 and PVX/SlCMT3 should target their intended genes for gene-specific VIGS.

Indeed, Cnr fruits undergoing VIGS of SlDRM7, SlMET1, SlCMT2 and SlCMT3 ripened to various degrees (Fig. 1c–e, Supplementary Fig. 3a–n). Particularly VIGS of SlCMT3 by PVX/SlCMT3, targeting the coding region of SlCMT3 mRNA, caused Cnr fruits to reach the stage of losing chlorophyll (equivalent to breaker) approximately 4 days earlier than Cnr fruits mock-inoculated with TE buffer or injected with PVX (Supplementary Fig. 4). SlCMT3-silenced fruits continued to ripen almost completely (Fig. 1f, Supplementary Fig. 5a–h). PVX/SlCMT3 targeting the 3’-UTR of SlCMT3 mRNA could also trigger Cnr fruit ripening (Fig. 1g, Supplementary Fig. 6a–i). However, not all CMT genes are necessary for maintenance of Cnr since SlCMT4 silencing had no effect on ripening (Fig. 1h, Supplementary Fig. 3o), further demonstrating that the observed ripening phenotypes were resulted from gene-specific VIGS by specific SlDMT constructs (Fig. 1b–i).

More than 60% of fruits at 5–15 days post anthesis were injected with PVX/SlCMT3, PVX/SlCMT3-UTR or PVX/SlCMT2 developed ripening phenotype. Only approximately 29% and 48% of fruits treated with PVX/SlMET1 or PVX/SlDRM7 appeared ripening. There was no ripening of Cnr fruits treated with PVX/SlCMT4, empty VIGS vector PVX, or mock-inoculated (Fig. 1i). It is worthwhile noting that no ripening was observed in rin fruits injected with PVX/SlCMT3 (Supplementary Fig. 3p). Taken together, our results

Figure 1 | SlDMT silencing causes Cnr epimutant to ripening. (a), Context, number and percentage of the hypermethylated cytosines (5°C) in the 286-bp LeSPL-CNR promoter region. (b), Diagram of VIGS vectors PVX/SlDRM7, PVX/SlMET1, PVX/SlCMT2, PVX/SlCMT3, PVX/SlCMT3-UTR and PVX/SlCMT4. (c–h), Ripening in Cnr fruits, assessed by red colour as compared to wild-type fruits (AC, (e)). No ripening was observed in fruits mock-inoculated (mock), inoculated with PVX or PVX/SlCMT4 (h). Photographs were taken at the indicated day post-anthesis (dpa). Bar = 1 cm. (i), Number of ripening fruits out of total number of inoculated fruits from at least two independent experiments.
demonstrate that functional SIbDMTs in the RdDM and methylation maintenance pathways are required for maintain the somatic stability of the non-ripening Cnr phenotype in the natural epimutant.

Developmentally regulated SlCMT3 is likely the key modulator for maintaining the Cnr epi-allele. In Arabidopsis, CMT genes are predominantly associated with maintenance of cytosine methylation in transposable elements\(^{6,11,13}\). It is therefore surprising that silencing of SlCMT2 and SlCMT3 (a close relative of Arabidopsis CMT3) should rescue Cnr ripening. It is also intriguing that SlCMT3 silencing had a greater effect on reverting the Cnr phenotype than silencing of SIdDM7 (a homologue of the Arabidopsis de novo methyltransferase DRM2) or other SIbDMTs (Fig. 1). These phenotypic differences may be due to variations in VIGS efficiencies, as noted previously \(^{20,22}\). Alternatively, our results may suggest that SlCMT3 plays a more prominent role in maintaining epi-alleles such as Cnr than SIdDM7 and other SIbDMTs. This is consistent with a high frequency of CHG hypermethylation in the LeSPL-CNR epimutated-region (Fig. 1a), the maintenance of which mainly requires functional SlCMT3\(^{35}\). We interpret these data to mean that SlCMT3 is probably one of the key genetic regulators underlying the inheritable maintenance of Cnr epimutation.

This hypothesis is supported by the fact that SlCMT3 expression is subject to developmental regulation. Expression of SlCMT3 changed dramatically in developing Cnr fruits, being extremely high at the immature stage then declining in mature green fruits (Fig. 2). The levels of SlCMT3 expression in immature Cnr fruits are so high that they dwarf those at all other stages of fruit development in normal and Cnr fruits (inset panels, Fig. 2). The SlCMT3 transcripts were again up-regulated in fruits at breaker before declining to lower levels in later stages. Expression of SlCMT3 in normal fruits was highest in green stages, but significantly lower than in immature Cnr fruits, and was down-regulated at breaker stage (Fig. 2). The prominent quantitative differences in expression of SlCMT3 between wild-type and Cnr fruits suggest that high level expression of SlCMT3 may be associated with the maintenance of the Cnr epi-status.

Silencing of SlCMT3 enhances LeSPL-CNR and other key ripening TF gene expression. To dissect the mechanism by which SlCMT3 repression causes the reversion of the Cnr to ripening, we analyzed whether SlCMT3 silencing affects expression of LeSPL-CNR and other key ripening transcription factor (TF) genes including LeMADS-RIN, LeHB1, SlAP2a, and SITAGL1\(^{24,25}\). Viral RNA declined dramatically in PVX/SlCMT3-injected fruits (Fig. 3a) and the silencing trigger SlCMT3 RNA was detected (Fig. 3b). Endogenous SlCMT3 mRNA in ripening pericarps was significantly reduced although only a moderate decrease was observed in the weakly ripe tissues of the same fruits (Fig. 3c, Supplementary Fig. 7a). In contrast with the reduction of SlCMT3 mRNA in silenced fruits, LeSPL-CNR was up-regulated when compared to levels in the control (Fig. 3d, Supplementary Fig. 7b). LeMADS-RIN, SlAP2a and SITAGL1 were also up-regulated, although LeHB1 expression was not significantly affected (Fig. 3e–h, Supplementary Fig. 7c–f). It should be noted that all TFs tested are known to be developmentally regulated in normal and Cnr fruits, although their expression levels differ and are generally much lower in Cnr\(^{24,25}\) (Supplementary Fig. 7g–h). These results demonstrate that Cnr-to-ripening reversion by SlCMT3 silencing is inversely correlated not only to the expression of LeSPL-CNR, but also to that of other ripening-associated TF genes. However, how VIGS of SlCMT3 influences expression of additional ripening TF genes remains to be elucidated. It is possible that such an impact could be a secondary effect of ripening or the change of the LeSPL-CNR expression, or/and is due to altered methylation of promoters of these TF genes.

Silencing of SlCMT3 enhances expression of genes involved in the biosynthesis and signal transduction of the ripening hormone ethylene. We also examined the expression of ethylene biosynthesis genes SIAC5, SIAC5, SIAC5, and SIAC5, and two ethylene signal transduction genes SIEF1 and SIEF2 during ripening of Cnr fruits. Consistent with up-regulation of ripening-associated TF gene expression, these ripening hormone-related genes were all found to be up-regulated in the ripe pericarp tissues in which SlCMT3 was silenced (Fig. 3i–m, Supplementary Fig. 8a–f). Indeed TFs such as LeMADS-RIN are known to regulate the expression of ethylene biosynthetic genes\(^{25}\). It is also possible that SlCMT3 is involved in the epigenetic regulation of these genes because levels of DNA methylation in their promoter regions in SlCMT3-silenced fruits were reduced, or that their up-regulation is the direct or indirect down-stream effect of LeSPL-CNR.

Silencing of SlCMT3 reduces cytosine methylation in the epimutated region of the LeSPL-CNR promoter. Targeted-bisulfite sequencing\(^{26}\) was used to examine methylation in the 286-bp region, and its flanking sequences, of the LeSPL-CNR promoter in the SlCMT3-silenced epi-allele fruits. A marked reduction of methylation was observed at eight specific cytosines, seven at the CHG sites and one in the CG context among the eighteen cytosine residues that are fully methylated in Cnr (Fig. 4a; Supplementary Fig. 9a–i). No clear difference in methylation was observed up- and downstream of the 286-bp region. These results indicate that the hypermethylation status of the eight cytosines is critical for inhibition of the LeSPL-CNR promoter activity, and the reduction in methylation of these residues may allow an increase in LeSPL-CNR expression; resulting in the "Cnr-to-ripening" reversion in the epimutant fruits. Taken into account of the gene-specific VIGS (Fig. 1, Supplementary Table 1, Supplementary Figs. 3, 5, 6), the effect of SlCMT3 reduction on the eight specific cytosine residues seems to refine the Cnr epi-allele in terms of functional hypomethylation.

Effect of SlCMT3 silencing on whole-genome DNA methylation. The single-base resolution methyleome of the SlCMT3-silenced Cnr fruit was further profiled by whole-genome bisulfite sequencing (WGBS), and confirmed the loss of methylation at the eight specific cytosines in the 286-bp promoter region (Fig. 4a, b). Moreover we observed that genome-wide hypomethylation occurred at CHG as
well as CG and CHH sites in repeats and gene regions (Fig. 4c–e). It is unlikely that the occurrence of hypomethylation at CG and CHH sites was due to non-specific silencing of other DMT genes by PVX/SlCMT3-mediated VIGS (Fig. 1, Supplementary Table 1, Supplementary Figs. 3, 5, 6, 10), although the underlying mechanism for such reduction of methylation requires further investigation. On the other hand, it has been well-documented that LeMADS-RIN is required for the activation of fruit ripening genes by directly binding to promoters of those genes\textsuperscript{21,28,29}. It has also been shown that LeMADS-RIN binding sites are demethylated in normal fruit and that LeMADS-RIN is unable to bind to the same sites in Cnr fruit due to a higher methylation level at those binding sites in Cnr than normal fruit\textsuperscript{29}. We thus examined the methylation levels of LeMADS-RIN binding sites in our WGBS data and found that these sites became hypomethylated after SlCMT3 silencing (Fig. 4f). These findings suggest that SlCMT3 loss-of-function not only disrupted the Cnr epi-allele but might have also helped to elevate LeMADS-RIN expression (Fig. 3e, Supplementary Fig. 7c) that would allow functional restoration of the LeMADS-RIN activity for binding to these demethylated sites.

Figure 3 | SlCMT3 affects expression of LeSPL–CNR and ripening genes. (a), PVX RNA. (b), Silencing trigger RNA (PVX-SlCMT3). (c–n), Endogenous SlCMT3, LeSPL-CNR, LeMADS-RIN, LeHB1, SIAP2a, SITAGL1, SIACS1, SIACS2, SIACS4, SIACO1, SIEBF1 and SIEBF2 mRNAs in non-ripening fruits mock-inoculated (Mo), inoculated with PVX, or in red-ripening (RR) and weak-ripening (WR) sectors of Cnr fruits inoculated with PVX/SlCMT3 (SlCMT3) at 31 days post inoculation. The inset-figure in (a) shows a low level of PVX RNA. Asterisk (*) indicates statistical significance (p < 0.001) by Student’s t-tests between the SlCMT3-silenced and PVX control samples.
Discussion

We describe a mechanism that maintains the stability of a naturally occurring epimutation, and thus of its associated phenotype in tomato. This mechanism relies on \textit{SICMT3}, possibly along with other key components such as \textit{SlDRM7}, \textit{SlCMT2}, and \textit{SlMET1}, in the RdDM and methylation maintenance pathways\textsuperscript{6–14}. Silencing of \textit{SlCMT3} in the epimutant fruits reduces methylation of eight specific cytosines mostly in the CHG context in the region of the \textit{LeSPL-CNR} promoter and causes genome-wide hypomethylation, resulting in an up-regulation of \textit{LeSPL-CNR} and key ripening genes and “\textit{Cnr}-to-ripening” reversion.

It is possible that the epi-allele \textit{LeSPL-CNR} and key ripening-associated transcription factor (TF) genes including \textit{LeMADS-RIN}, \textit{SlAP2a}, and \textit{SlTAGL1} form a regulatory network that controls tomato development and fruit ripening. These TFs can regulate each other and they are involved in possible feedback loops in the genetic regu-

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Figure 4 | Analysis of single-base resolution methylome. (a–b), Targeted and whole-genome bisulfite sequencing (TBS, WGBS) reveals methylation changes in specific cytosine residues (a) and the overall \textit{Cnr} promoter region (b) in the \textit{SICMT3}-silenced \textit{Cnr} fruit. Bar-chart shows the methylation levels in the \textit{Cnr} gene locus in epimutant fruit at breaker stage (\textit{Cnr}), \textit{SICMT3}-silenced \textit{Cnr} fruit at breaker stage (VIGS), and in wild-type fruit at immature (IM), mature green (MG), breaker (Br), ripening stages (Ripen), and \textit{LeMADS-RIN} ChIP-Seq (RIN binding). The location of the two differentially methylated regions (DMR1 and DMR2) and the epi-allele in the promoter region of \textit{Cnr} are shown. (c–d), Genome-wide hypomethylation caused by \textit{SICMT3} silencing. Kernel density plots of the loss of CG (c), CHG (d) and CHH (e) methylation in the \textit{SICMT3}-silenced \textit{Cnr} fruit at breaker stage. Methylation differences (methylation level of \textit{Cnr} minus \textit{SICMT3} silenced \textit{Cnr} fruit at breaker stage) of the whole-genome (bin = 1000 bp), annotated gene regions, repeats, and the \textit{LeMADS-RIN} bindings sites are shown, and regions with zero methylation are discarded\textsuperscript{29}. (f), \textit{SICMT3} silencing causes global demethylation in \textit{Cnr} fruit. Boxplot showing the delta-methylation levels of \textit{Cnr} and \textit{SICMT3}-silenced fruits at the breaker stage. For calculation of the global methylation delta, genome is divided into 200-bp bins and the methylation levels of each bin are calculated. Gene and the repeat are defined according to the ITAG v2.5 annotation. RIN binding sites are called as previously described\textsuperscript{29}. 

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of ripening\textsuperscript{25–28}. TFs also regulate fruit ripening via transcriptional regulation of ethylene biosynthesis and signal transduction. Regulation of \textit{LeSPL-CNR} expression by SlymiRNA157 is also incorporated into this model. Blue arrow indicates activation while the ‘T’ sign represents inhibition. Grey arrow and ‘T’ sign indicate potential functional mode.

Figure 5 | Maintenance of epigenetic stability in regulating tomato fruit ripening. \textit{LeSPL-CNR} and key ripening-associated transcription factor (TF) genes form a regulatory circuit in the genetic and epigenetic control of tomato fruit ripening via modulation of ethylene biosynthesis and signal transduction. Regulation of \textit{LeSPL-CNR} expression by SlymiRNA157 is also incorporated into this model. Blue arrow indicates activation while the ‘T’ sign represents inhibition. Grey arrow and ‘T’ sign indicate potential functional mode.

Methods

Constructs. Non-translatable 300–525-bp fragments corresponding to the 5’ ends of each gene were PCR-amplified and cloned into the MuI/SeII sites of the \textit{Potato virus X} (PVX)/\textit{SdM1} vector\textsuperscript{e} to generate PVX/\textit{SdM1}, PVX/\textit{SdM2}, PVX/\textit{SICM3}, and PVX/\textit{SICM4} (Fig. 1b). The 3’ UTR of the \textit{SICM3} was also cloned into PVX to produce PVX/\textit{SICM3UT}. The full-length cDNA sequences of the nine tomato DMT genes and the sequences of the short non-translatable fragments that were used for construction of the PVX-based VIGS constructs are included in Supplementary Figure 10. A non-translatable \textit{LeSPL-CNR} gene and the 286-bp region of the \textit{LeSPL-CNR} promoter were cloned into the PVX/GFP vector\textsuperscript{e} to generate PVX/m\textit{LeSPL-CNR-GFP} and PVX/Pcnr-GFP (Supplementary Fig. 1a). PVX encodes a RNA-dependent RNA polymerase (166 K), movement proteins (25 K, 12 K and 8 K) and capsid protein (CF). Primers are listed in Supplementary Table 2. All constructs were confirmed by sequencing.

PVX-based gene silencing and plant growth conditions. PVX-based VIGS and virus-induced transcriptional gene silencing in \textit{Cnr}, \textit{rin} and wild-type tomato (\textit{Solanum lycopersicum} cv. Adisa Craig) fruits were performed as described\textsuperscript{31–32}. The carpodium of tomato fruits at 5–15 days post anthesis was needle-injected with recombinant viral RNAs for each of the PVX-based VIGS constructs. Plants were grown in insect-free glasshouses at 25°C with supplemental lighting to give a 16-h photoperiod, examined and photographed with a Nikon Coolpix 995 digital camera.

Quantitative real-time PCR (qRT-PCR). Total RNA was extracted from tomato tissues using RNasey Plant Mini Kit (Qiagen). cDNA was synthesized using a FastQuant RT Kit (Tiangen). qRT-PCR was performed on a Bio-Rad CFX96 Real-Time system (Bio-Rad) using an UltraSYBR Mixture Kit (CoWin Bioscience). At least three technical replicates for each of three biological replicates for each sample were analyzed. The relative level of specific gene expression was calculated using the formula \(2^{\Delta \Delta C_{t}}\) and normalized to the amount of 18S rRNA detected in the same sample as described\textsuperscript{33, 34}.

Bisulfite sequencing. Total DNA was isolated from tomato tissues using DNeasy Plant Mini Kit (Qiagen). Bisulfite conversion, PCR amplification and sequencing were performed using the EZ DNA Gold Methylation Kit (Zymo Research), Blue MegaMix Double PCR mixture (Microzone) and BigDye Terminator Reaction Mixture (Applied BioSystems) as described\textsuperscript{35, 36}. Whole genome bisulfite sequencing and bioinformatics analysis were performed as previously described\textsuperscript{37–38}.

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Author contributions
W.C., J.K., C.Q. and Y.H. designed and performed experiments; J.T., C.W., H.W., Y.S., C.L., B.L., P.Z., Y.W., T.L., Z.Y., X.Z. and N.S. performed experiments; Y.C., S.Y. and S.Z. performed the WGBS and analysed the data; H.-z.W., T.O., Y.L., K.M., S.I., D.R., S.Z. and G.B.S. were involved in discussions and helped writing the paper; P.G. analysed data and helped writing the paper; Y.H. initiated the project, analysed data and wrote the paper.

Additional information
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