Molecular cloning and characterization of GbMECT and GbMECP gene promoters from *Ginkgo biloba*

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ABSTRACT. Ginkgolides are key pharmaceutical components in *Ginkgo biloba*. Using the cDNA sequence of the MECP and MECT genes to design primers, we obtained the promoters of these genes from *Ginkgo* genomic DNA using the genome walking method. The two promoters were 744 and 982 bp in length, respectively. The cis-elements of the GbMECPs and GbMECT promoters were predicted and analyzed using the plant cis-acting regulatory element database. We found major cis-elements in the sequence of the GbMECT and GbMECPs promoters. The GbMECP promoter contains six TATA boxes and eight CAAT boxes. The GbMECT contains five TATA boxes and seven CAAT boxes. Furthermore, some cis-elements in the promoters of GbMECPs and GbMECT included hormone and light-regulated elements, UB-B-induced elements, and stress-related dehydration-responsive elements. Expression analysis results showed that the MECP gene is mainly involved in responses to CCC (cycocel) and UV-

B, and that MECT is mainly involved in responses to wounding treatment. These results also showed that the expression model was consistent with the cis-elements present. During the annual growth cycle, the level of GbMECPs was significantly correlated with terpene lactones accumulation in leaves. A fitted quadratic curve showed the best model for correlating GbMECPs with terpene lactones in leaves. These results will help us to understand the transcriptional regulatory mechanisms involved in key gene expression and ginkgolide accumulation in *G. biloba*.

INTRODUCTION

In recent years, *Ginkgo biloba* has become one of the most popular functional plants, especially for medicinal purposes. Extracts of *G. biloba* leaves contain active compounds such as flavonoids and terpene lactones (especially ginkgolides and bilobalide), and can, therefore, be used to increase peripheral and cerebral blood flow (Smith and Luo, 2004). Terpenoids are the most structurally diverse group of plant natural products (Kang et al., 2013). Although ginkgolides are considered to play a key role in the active ingredients of ginkgo, the concentration of ginkgolides is very low, at 0.25% in leaves (van Beek and Montoro, 2009). Therefore, terpene trilactone content should be increased in order to improve the medicinal and commercial value of ginkgo leaf production.

Plants use both the classical mevalonic acid (MVA) pathway and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway for isoprenoid biosynthesis, although they are localized in different compartments (Lichtenthaler, 1999). Anabolism of ginkgo terpene trilactones occurs mainly through the MEP pathway. The enzyme involved in the fifth step of the MEP pathway is 2-C-methylerythritol 2,4-cyclodiphosphate synthase, which is encoded by the Mecps gene (Bick and Lange, 2003). Experimental data have revealed that MECPs participates in the control of isoprenoid accumulation in plants, A correlation between MECPs expression and terpenoid accumulation has also been demonstrated (Veau et al., 2000). 2-*C*-methyl-D- erythritol 4-phosphate Cytidyltransferase (MECT) is the third enzyme in the MEP pathway, which catalyzes the formation of 4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol from MEP and has been cloned and characterized from *G. biloba* embryonic roots (Kim et al., 2006). The expression of MECP and MECT genes is elaborately regulated by its growth as well as internal and external factors, as evidenced by the complex interaction between transcription factors and acting elements. Interaction between the expression of regulatory elements upstream of the two genes and trans-acting factors clearly merits further investigation as it will help regulate the accumulation of Ginkgo terpene. In this study, the upstream transcription regulatory sequence of the two genes, *GbMECP* and *GbMECTs*, were cloned via genome walking technology and bioinformatics analysis was performed. The two genes expression model were detected under different stress treatments, and also evaluated the relationship with terpene lactone in annual growth year in Ginkgo leaves. Studies investigating the role of the regulatory sequences in structural gene expression during synthetic metabolism in ginkgolides are crucial for identifying the molecular mechanisms involved in this process.

MATERIAL AND METHODS

Plant material and treatments

Twelve-year-old grafted *G. biloba* seedlings were growing in a greenhouse in Huanggang (E, 114°54'-116°8', N, 29°45'-31°35', Hubei province, east of China) were sampled and used to isolate GbMECPs and MECT promoters. Seedlings from the same genotypic strain of *G. biloba* were subjected to treatments with UV-B, wounding (WOU), and chlormequat (CCC). For UV-B treatment, seedlings were exposed to 1500 J/m² UV-B irradiation in a closed chamber, and the control cuttings were placed in a dark closed chamber. The edges of leaves were wounded by cutting ~0.5 cm with scissors, the intact leaves of seedling were used as the control. CCC (10 mM) was dissolved in 0.01% Tween 20 and sprayed onto cutting leaves. The control leaves were sprayed with an equivalent volume of 0.01% (v/v) Tween 20. All samples were immediately frozen in liquid nitrogen and stored at -80°C prior to RNA extraction and gene expression analysis. The different oligonucleotides used in this work are described in Table 1.

Table 1. Genomewalker and expression analysis primers.

Description	Serial number	Sequences (5'-3')
Adaptor primers	AP1	GTAATACGACTCACTATAGGGC
	AP2	ACTATAGGGCACGCGTGGT
Primers for promoter isolation	MT1	GTGAGGAGACAATGAACTGGGAAC
	MT2	CTGGGAACGGTGTTACATGCTA
	MT3	CATCGTCATCATCTTCATAGACTTCGGC
	MT4	TAAGGACAATAGGAGGAAAAGTA
	MPS1	CTGATGA TTATGAACCCAACTGATT
	MPS2	CAACTGATTGCAGATTTGAGAGAT
	MPS3	GGTC TCGCT TATGCGGTTCCATGTTCC
	MPS4	TAGTTAGGTATCAAGGTCTCCGT
Primers for verification	MECT1	AATGCTTGTAGACTCTTCTTCTACTTTCC
	MECT2	ATGTCATGTCATTTGAGACTTACG
	MPC	GATCGATCTGGAACGATTGATGGAATCCGA
	MPB	AGGATCCACGCTCTTGCGGTCGTACTATGC
	MPU	CGAGTGCCAACGACCATGA AT
	MPD	ACCCACACTTTGCCGTAATGAG
Primer for RQPCR	MET1	TAAGGGTGCTGATTCTGGGACG
	MET2	TTCCTCCTGCCAATAGAACAAC
	MEP1	CAGTTTTCTTGAAGGAGGCGGT
	MEP2	TAGCCTCTTTATGGGGACTCAA
	GAPU	TAGGAATCCCGAGGAAATACC
	GAPD	TTCACGCCAACAACGAACATG

Relative quantification by qRT-PCR

The levels of GbMECT and GbMECP transcripts were determined in different *G. biloba* tissues, as well as in leaf samples from young seedlings collected at different time points after stress and hormone treatment. qRT-PCR was carried out using an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, USA) with SYBR Green PCR Master Mix (Applied Biosystems, USA) according to the manufacturer protocol. GbMECT and GbMECP sequences were obtained from NCBI database supplied by Kim(2006) and Gao(2006). The *G. biloba* glyceraldehyde 3-phosphate dehydrogenase gene (GbGAPDH, L26924) was used as the internal reference gene as described by Cheng et al. (2013).

Extraction and determination of Terpene lactones

Ginkgolide A, ginkgolide B(GB), ginkgolide C(GC), and bilobalide (BB) were extracted and their levels were determined using gas chromatography with a wide bore capillary column as Liao (2015) described. The total terpene lactone contents was determined as the sum of the Ginkgolide A, GB, GC, and BB in G. biloba, and sesquiterpene lactone calculated as the contents of BB. All tests were carried out in triplicate, and data represent the means ± standard errors.

DNA and RNA extraction

Genomic DNA was extracted from fresh leaves of *G. biloba* using the improved CTAB(Cetyltrimethyl ammonium bromide) method as described by Jiang (2000). Total RNA was isolated from different treatment of Ginkgo leaves using the method of Cheng(2013), followed by incubation with RNase-free DNase I (TaKaRa, Dalian, China) at 37°C for 30 min. The quality and concentration of genomic DNA and RNA were determined by agarose gel electrophoresis and spectrophotometer analysis, respectively.

Bioinformatics analysis

The obtained sequences were analyzed using online bioinformatics tools (http://www. ncbi.nlm.nih.gov). The sequence region was analyzed using the plant cis-acting regulatory element (CARE) database (http://www.dna.affrc.go.jp/PLACE and http:// bioinformatics.psb.ugent.be/ webtools/ plantcare/html/).

RESULTS

Identification and characteristics of MECT and MECPs specific promoters

Promoter element analysis showed that Ginkgo MECPs and MECT gene promoter sequences contain a large number of cis-acting elements (Figure 1, Table 2). Therein, the basal promoter element, TATA box, is located 28 bp upstream of the transcription start site of the MECPs gene. The CAAT box is located -53 bp upstream of the transcription start site and the MECT is located between -106 and -150 bp. In addition, there are a further five TATA boxes and seven CAAT boxes in the promoter, indicating that it had strong transcription signals. Table 1 summarizes the different components contained within the promoter fragment, and the results of the preliminary forecast are as follows: GT-1 motif (GRWAAW) (Terzaghi and Cashmore, 1995) contains seven MECPs and MECT, and it is a conserved regulatory site in light-regulated genes; MECPs has three Circadian boxes (CAANNNNATC) (Piechulla et al., 1998), while MECT has none, which is a typical response element to circadian rhythms; GATA box (GATA) (Gilmartin et al., 1990), I-box (GATAA) (Terzaghi and Cashmore 1995) represent the acting elements that are closely related to light response and regulation. As for the acting elements associated with hormone responses, there is one main auxin response element GH3 (CACGCAAT) (Ulmasov et al., 1995), one gibberellin regulatory element GARE-motif (TAACAGA) (Sutoh and Yamauchi, 2003), three WRKY-motifs (TGAC) (Zhang et al., 2004), one ethylene-responsive element ACS (TAAAATAT) (Matarasso et al., 2005), and 15 elements related to cytokinin ARR1 (NGATT) (Ross et al., 2004), one CPBCSPOR (TATTAG) (Fusada et al., 2005), one abscisic acid response element ABRE (ACGTG) (Nakashima

et al., 2006), and two Dc3 elements (ACACNNG) (Kim et al., 1997). Furthermore, regulatory elements that are associated with stress include MYB and MYC transcription factor binding sites, including five MYB1AT(WAACCA) (Abe et al., 2003), two MYBCORE(CNGTTR) (Urao et al., 1993), one ATMYB2 (CTAACCA) (Abe et al., 1997), one MYCATRD22 (CACATG) (Chinnusamy et al., 2003), and 18 CBF3 (CANNTG) (Rieping and Schöffl, 1992). These regulatory elements are mainly related to environmental stimuli such as dehydration, drought, and cold resistance; MYB and MYC may mediate stress responses by acting as transcription factors. The sequence has been found to contain a high temperature stress response element HSE-motif (CCAAT) (Rieping and Schöffl, 1992) and the cis-acting elements associated with the biological disease As-1 (TTGAC) (Chen and Chen 2002).

In addition to elements related to light, hormones, and adverse stress, the GbMECT promoter sequence also contains the following specific elements: eight root tissue-specific elements rolD (ATATT) (Elmayan and Tepfer 1995), which are located at -797, -487, -298 , -221, -214, -181, -160, and -137 bp; elements related to phenylalanine and lignin metabolism MYBPLANT (MACCWAMC, -685 bp) (Tamagnone et al., 1998); sugar inhibition elements SRE (TTATCC) (Tatematsu et al., 2005) located at -595 bp; oxidative stress and the copper ion response element CuRE (GTAC) (Quinn et al., 2002) located at -414 bp; and an amylase response element amylase-box (TATCCAT, -594 bp) (Hwang et al., 1998) .

Figure 1. Nucleotide sequence (part) and in silico prediction of TBFs of the GbMECT and MECP promoter region.

Response of MECT and MECPs-specific promoters to wounding, CCC, and UV-B

To confirm that gene expression was regulated by regulatory elements in the promoter region, we investigated the response to three treatments: WOU, CCC, and UV-B, and employed qRT-PCR to test the influence of these treatments on MECT and MECPs gene transcription (Figure 2). The results show that all injurious treatments led to abnormal expression of the MECT and MECP genes, and were found to significantly induce MECT gene expression. The highest expression of each gene was observed after 12 h, and then decreased slowly. CCC treatment significantly induced MECP gene expression, and its transcriptional level was maximal 12 h after treatment, after which it declined. CCC treatment had no significant effect on MECT gene expression. MECP was strongly induced under the UV-B treatment, and expression increased rapidly after 4 h, decreased at the 8 h sampling point, and reached the highest level at 12 h before a rapid decline thereafter. Conversely, the effect of UV-B on MECT induction was slow, and expression increased slightly at 8h, and was ~1.2-fold higher at 12 h, which was maintained thereafter.

Table 2. Cis-acting elements analysis of promoter sequence from Ginkgo biloba GbMECPs and MECT by PlantCARE.

Element type	Name of element	Motif Sequence	Copy number in promoter		Function
			MECPs	MECT	
Light response cis-element	R2R3type	ACCWWCC	1		DCMYB1 bound to these sequences; PAL: elicitor; UV-B; dilution
	Circadian	CAANNNNATC	3		Region necessary for circadian expression
	GATA box	GATA	9	7	light regulation, and tissue specific expression
	GT-1	GRWAAW	7	7	Consensus GT-1 binding site in many light-regulated genes
	I-box	GATAA	4	2	Light-regulated transcription
	ASF-1motif	TGACG		2	Light-regulated transcription like I-box
	Inr-element	YTCANTYY	4	2	initiator; light-responsive transcription
Hormone reponse cis-element	ABRE	ACGTG	1		Transcriptional regulation of ABI3 and ABA-responsive genes
	ARR1	NGATT	15	8	Response regulator
	Dc3	ACACNNG	2		interacts with ABA-responsive and embryo -specification elements in the Dc3 promoter
	MYC	CANNTG		6	Transcriptional regulation of ABI3 and ABA-responsive genes
	E-box	CANNTG	18	6	R response element
	GARE	TAACAGA	1		gibberellin-upregulated proteinase expression
	ACS	TAAAATAT	1		Cysteine protease; ethylene; xylanase
	WRKY	TGAC	3	1	A transcriptional repressor of the gibberellin signaling pathway
	CAREs and CARE	CAACTC		3	Gibberellin-upregulated proteinase expression
	GH3	CACGCAAT	1		Composite structure of auxin response elements
	CPBCSPOR	TATTAG	1	1	Identification of a Novel Cis-Element Exhibiting Cytokinin-Dependent Protein
	GA-element	TAACAAR		3	Gibberellin biosynthesis and response
	TATCCA-element			2	Gibberellin Response Element
	ASF-1motif	TGACG		1	Auxin; salicylic acid; Disease resistance
Stress inducible cis-acting	HSE	CCAAT		1	Cis-acting element involved in heat stress responsiveness
	LTRE-1	CCGAAA	1		Identification of promoter elements in a low- temperature-responsive gene
	rd22BP1	WAACCA	1		MYB recognition site found in the promoters of the dehydration-responsive gene rd22
	ATMYB2	CTAACCA	5		Dehydration; Water stress; ABA; MYC
	W-box	CTGACY	1	1	Elicitor-responsive transcription of defense genes
	MYBCORE	CNGTTR	2		Binding site for all animal MYB, responsive to water stress
	MYCATRD22	CACATG	1		Binding site for MYC, dehydration-responsive gene
	CBF3	CANNTG	18		Binding site of ICE1, a regulator of cold-induced transcriptome and freezing tolerance
	TGTCA	TGTCA		1	Disease resistance responses
	ACS	TAAAATAT		1	Cysteine protease; ethylene
	As-1	TTGAC	2		Disease resistance
	ERF3	TGACY	3	1	May be involved in ERF3 gene activation in response to wounding
Others cis-element	CATGTG motif	CATGTG	1		drought-responsive cis-element in the early responsive to dehydration stress
	AMYbox1	TAACARA	1		Conserved sequence found in 5'-upstream region of alpha-amylase gene
	CACT	YACT	3		cis-Regulatory elements for mesophyll-specific gene expression
	napA	CNAACAC	1		Identification of promoter elements required for embryo and endosperm-specific transcription
	Dof	AAAG	10		Transcription factors
	GTGA-motif	GTGA	8		Functional analysis of cis-regulatory elements
	MYBPLANT	MACCWAMC	1		The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis

Relationship between the MECT and MECPs genes and levels of terpene lactones

To study the relationship between terpene content and the expression of two genes of interest, annual changes between the two parameters measured during the growth of Ginkgo leaves were investigated. Grafted Ginkgo leaves were collected at seven sampling points (May12; June 13; July 18; August 13; September 11; October 17; November 14, 2013) for RT-PCR analysis and to measure the terpene lactone content. Figure 3 shows changes in the sesquiterpene content in *G. biloba* under natural growth conditions. From May to August, the sesquiterpene content in *G. biloba* increased slowly, and quickly rose to the highest annual level from August to October (5.05 mg/g). Figure 4 demonstrates the changing content of total triterpenoids from *G. biloba*. From May to July, the total triterpenoid content rapidly rose and peaked at ~7.9 mg/g in August, decreased rapidly to 6.7 mg/g in September, and rose to the highest annual level of 8.67 mg/g in October, before decreasing slightly to 7.71 mg/g in November.

However, the changes in gene expression varied considerably between MECPs and MECT. Expression of *MECP* declined slightly from May to June and increased afterwards until the highest annual level of 6.47-fold was reached in October, but fell sharply in November to 4.25-fold. Expression of *MECT* increased rapidly from May to July, a 6.7-fold increase, subsequently declining rapidly to the lowest level of 3.48-fold in November.

A curve-fitting model was implemented between the annual periodic changes and gene expression and total terpenoids. There was a significant quadratic correlation between the level of *MECP* transcription and the content of terpene lactones in *G. biloba*, with a correlation coefficient of 0.862 and a correction coefficient of 0.714 (Table 3). The relevant equation is as follows: $Y = (0.999 \pm 0.155)X^2 - (10.372 \pm 1.670)X + (30.443 \pm 4.453)$, where Y is the change in total terpene lactones content and X is the level of *MECP* transcription (Table 4 and 5). But there was a weak correlation between MECT and terpene lactones content in leaves. The regression analysis result showed the two genes have no obvious relationship with the sesquiterpenes contents in annual growth year.
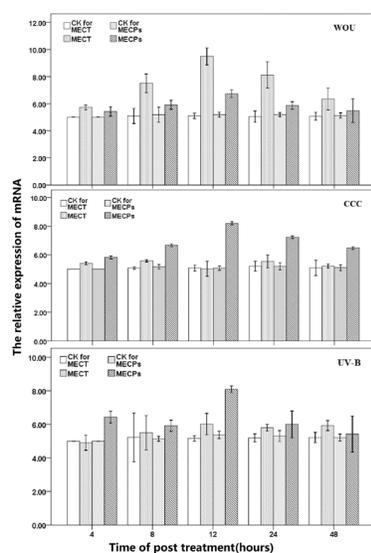
Figure 3. Relationship between the relative expression of *MECPs* and *MECT* and the diterpenoid content in Ginkgo leafs by month.
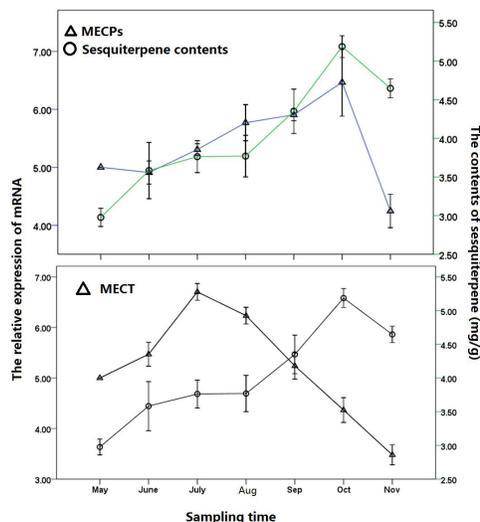
Figure 4. Relationship between the relative expression level of *MECPs* and *MECT* and the terpene lactone content in Ginkgo leafs by month.

Table 3. Coefficient change of quadratic model determination.

R	R Square	Adjusted R Square	Std. Error of the Estimate
0.862	0.742	0.714	0.377

The independent variable is MECP.

Table 4. Inspection of the quadratic model variance analysis.

	Sum of Squares	Df	Mean Square	F	Sig.
Regression	7.367	2	3.683	25.915	0.000
Residual	2.558	18	0.142		
Total	9.925	20			

The independent variable is MECP.

Table 5. Coefficient of the test results of the models between GbMECP and terpene lactones contents.

	Unstandardized coefficients		Standardized coefficients	*t*	Sig.
	B	Std. Error	Beta		
MECP	-10.372	1.670	-10.402	-6.212	0.000
MECP**2	0.999	0.155	10.811	6.456	0.000
(Constant)	30.443	4.453		6.836	0.000

DISCUSSION

Secondary metabolism in plants is affected not only by primary metabolism, but also by the direct regulation of environmental factors (Jacobs and Browner, 2000). Ginkgolides are secondary metabolites in plants, and their level is influenced by integrated conditions including

illumination, temperature, and soil moisture. Changes in the external environment may also have an impact on the synthesis of endogenous plant hormones such as auxin, gibberellin, ethylene, and ABA (abscisic acid). Some hormones can induce or inhibit the expression and transcription of genes related to ginkgolide synthesis, by affecting the rate or direction of certain precursors in the ginkgolide biosynthesis as well as affecting the ginkgolide content.

The influence of light conditions on biosynthesis and accumulation of ginkgolides has been rarely reported. Leng (2002) explored the effects of light quality and intensity on ginkgolide levels, and found that they are significantly affected by light quality. Whether this is because light quality promotes the expression of genes related to ginkgolide synthesis remains unclear. The ultraviolet-responsive element ACCWWCC present in the GbMECPs promoter sequence also exists in the carrot PAL1 promoter sequence, and it has been found that after UV-B irradiation, DCMYB1 can promote PAL1 gene expression by binding to this conserved sequence (Maeda et al., 2005). In addition to the light regulatory conserved element GT-1 (GRWAAW), the Gingko MECPs gene promoter also contains a cis-acting element that is responsive to circadian rhythms, the Circadian box (CAANNNNATC), the presence of which has been confirmed in *arabidopsis* and other plants (Wan et al., 2010). Hence, expression of *MECP* induced by UV-B should be related to a regulatory response involving the UV-responsive element ACCWWCC.

Studies have shown that exogenous CCC can significantly induce the expression of four key genes (DXS, DXR, GGPPS, LPS) involved in the biosynthesis pathways of ginkgolides, indicating that CCC may promote the biosynthesis of ginkgolides by inducing the upregulation of key gene expression at the molecular level (Xu et al., 2011). Analysis of expression in ginkgo showed that CCC treatment could also significantly induce the transcriptional upregulation of *MECPs*. Seasonal analysis of sesquiterpenes in *G. biloba* revealed that the level of *MECPs* transcription was significantly correlated with sesquiterpenes and total terpene lactones; therefore, we speculate that *MECPs* is a key gene involved in ginkgolide biosynthesis.

When tobacco plants are injured exogenously, the WRKY gene quickly binds to W-box elements and induces the expression of related genes (Nishiuchi et al., 2004). GbMECT contains one significant W-box, which is not present in GbMECP; this is consistent with our results showing increased MECT gene expression following wounding. It is therefore presumed that the increase in ginkgolides in response to injury is closely related to the expression of *GbMECT.* Mild drought leads to the accumulation of ginkgolides in leaves and roots, and the HMGR and MVD genes regulate the metabolism of BB in the MVA pathway, whilst DXS, DXR, MECT, and MECPs regulate total diterpene lactones biosynthesis in the MEP pathway (Zhu, 2010). It is speculated that the environment stimulate the transcription level of MECPs gene to improved the diterpene lactones biosynthesis in MEP pathway. Table 2 shows that *GbMECPs* and *GbMECT* contain multiple regulatory elements, including MYB1AT, rd22BP1, ATMYB2, and MYCATRD22, which are stimulated by water shortage. We speculate that MYB and MYC act as transcription factors to mediate adversity stress like drought (Abe et al., 1997). Therefore, these regulatory elements may be involved in the drought stress response of the GbMECPs and GbMECT genes. Functional research, and investigation of seasonal changes in GbMECPs and GbMECT expression and promoter analysis involved in ginkgolide biosynthesis will help us understand how ginkgolide biosynthesis is induced by the environment.

Conflicts of interest

The authors declare no conflict of interest.

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