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Quantification of Genetically Modified Canola GT73 Using TaqMan Real-Time PCR

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Abstract Event-specific PCR detection methods are the primary trend in genetically modified (GM) plant detection owing to their high specificity based on the flanking sequence of the exogenous integrant. Therefore, this study describes a real-time PCR system for event-specific GM canola GT73, consisting of a set of primers, TaqMan probe, and single target standard plasmid. For the specific detection of GT73 canola, the 3'-integration junction sequence between the host plant DNA and the integrated specific border was targeted. To validate the proposed method, test samples of 0, 1, 3, 5, and 10% GT73 canola were quantified. The method was also assayed with 15 different plants, and no amplification signal was observed in a real-time PCR assay with any of the species tested, other than GT73 canola.

Key words: EPSPS, genetically modified canola, GOX, GT73, real-time PCR

Oilseed rape (*Brassica napus* L.) is grown as a commercial crop in 50 countries with a combined harvest of over 40 million metric tons [1]. In additon, the use of canola oil is now widespread in the food industry as a vegetable oil for cooking, and as an ingredient in a range of mixed foods. However, since certain weeds are difficult to control when growing conventional canola, and growers tend to choose herbicide-resistant GM canola varieties that allow more effective weed control, thereby producing a better yield, better returns, and more profit [4]. GT73, a commercialized GM canola used worldwide, was made tolerant to the herbicide glyphosate using two trans-genes. The first trans-gene is the *epsps* gene coding the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), isolated from the common soil bacterium *Agrobacterium tumefaciens*, and is a glyphosate-tolerant form of EPSPS [12]. The second trans-gene in GT73 encodes a modified version of the glyphosate oxidase (GOX) enzyme and was isolated from the bacterium *Ochrobactrum anthropi*. The GOX enzyme accelerates the normal breakdown of the herbicide glyphosate into two compounds, aminomethylphosphonic acid (AMPA) and glyoxylate [2, 5, 13]. In the absence of GOX, unacceptable levels of the herbicide can accumulate in the canola cake in animal feed. The two trans-genes were introduced into GT73 in a plasmid using the bacterium *Agrobacterium tumefaciens*. The *epsps* and *gox* genes were each driven by the 35S promoter from a modified figwort mosaic virus and terminated with the 3' (terminal) end of the pea *rbcS E9* gene. The quantification of GM canola has been reported based on targeting canola endogenous genes, such as the high-mobility-group protein I/Y (*HMG-I/Y*) gene [18], phosphoenolpyruvate carboxylase (*pep*) gene [20], acetyl-CoA carboxylase BnACCg8 gene [8], and acyl-acyl carrier protein (*ACP*) thioesterase (*FatA*) gene [16], and targeting the event specific sequence of GM canola. For these quantitative real-time PCRs, a LightCycler 1.0 (Roche, Laval, Canada), ABI PRISM 7000 and 7700 (Applied Biosystems, Foster City, U.S.A.), and fluorometric thermal cycler (Rotor Gene 2000, Corbett Research, Melbourne, Australia) have all been used as the thermal cycler. Thus, given such a variety of detection methods, a standard method for the detection of GM canola is needed in Korea [6, 17].

Accordingly, in an effort to establish a standard method for the detection of GM canola in Korea, this study presents a real-time PCR method using recombinant p3'F-FatA (standard plasmid) containing FatA as the endogenous gene and event-specific sequence of GT73 for absolute quantitation. A TaqMan-based real-time PCR system was used, with an ABI PRISM 7900 (Applied Biosystems) as the thermal cycler, plus the detection limit, accuracy, and

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precision were all investigated using the standard plasmid and coefficient value.

MATERIALS AND METHODS

Materials

Roundup Ready GT73 (synonym RT73) (Monsanto, St. Louis, U.S.A.) GM canola and non-GM canola seeds were purchased from the American Oil Chemists' Society. Fifteen different crops, including rice (*Oryza sativa* L.), pepper (*Capsicum annuum* L.), corn (*Zea mays* L.), soybean (*Glycine max* (L.) Merr.), tomato (*Lycopersicon esculentum* var. *esculentum*), perilla (*Perilla frutescens* (L.) Britt.), sunflower (*Helianthus annuus* L.), cucumber (*Cucumis sativus* L.), buckwheat (*Fagopyrum esculentum* Moench), cotton (*Gossypium hirsutum* L.), mung beans (*Phaseolus radiatus* L.), barley (*Hordeum vulgare* L.), wheat (*Triticum aestivum* L.), Chinese cabbage (*Brassica rapa* subsp. *pekinensis* (Lour) Hanelt), and red-bean (*Phaseolus angularis* (Wild) W.F. Wight), were provided from the Rural Development Administration in Korea.

Oligonucleotide Primers and Probes

The nucleotide sequences of the primers and probes are shown in Table 1. The primers were synthesized and purified on PAGE columns at the Bionics Company (Seoul, Korea), whereas the TaqMan fluorescent dye-labeled probes were synthesized by Applied Biosystems. The probes were labeled with 6-carboxy-fluorescein (FAM) at the 5'-end and tetramethyl-6-carboxyrhodamine (TAMRA) at the 3'-end. The primer pair GT73-5/3 amplified a 108-bp product, and the primer pair Fat-FR1/RR1 amplified a 111-bp product. As such, the Fat-FR1/RR1 and GT73-5/3 primers detected a *FatA* gene as an endogenous reference control for canola and the 3'-flanking region as a positive control for the transgene in the GM canola, respectively. The Fat-FR1/RR1 and GT73-5/3 primers were also used in constructing the standard plasmid.

DNA Extraction

The genomic DNA of the canola and other crops was extracted from ground samples (about 1 g) using a DNeasy Plant Maxi kit (Qiagen GmbH, Hilden, Germany) according to a modified manufacturer's manual. The quality of the extracted DNA was evaluated using a UV spectrophotometer UV-1700 (Shimadzu, Kyoto, Japan) and analyzed by 1% agarose gel electrophoresis.

Standard Plasmid as Reference Molecule

A standard plasmid, a reference molecule with an endogenous *FatA* gene and exogenous fragment of the event-specific sequence in GT73, was constructed based on a Perfect-T cloning kit (TaKaRa, Kyoto, Japan) integrated with two PCR amplicons. The primer pairs Fat-FR1/RR1 and GT73-5/3 employed to clone the two fragments were designed based on amplified sequences of the *FatA* gene and 3'-flanking region in GT73, respectively. The PCR amplicons obtained using these primer pairs were then analyzed by 2% agarose gel electrophoresis and purified using a Gel Extraction kit (Qiagen). Thereafter, the PCR product was ligated into a pMD18-T vector (TaKaRa), and the recombinant plasmid transformed into *Escherichia coli* strain DH5α (Novagen, Madison, U.S.A.). The sequences of the cloned DNA were analyzed using an ABIPRISM 3700 DNA analyzer (Perkin Elmer, MA, U.S.A.) [7, 11].

According to the above procedures, the standard plasmid p3'F-FatA as the reference molecule was constructed by the tandem combination of two amplicons obtained using separate primer pairs for *FatA*, a canola endogenous gene, and the 3'-flanking region, an exogenous fragment in GT73 (Fig. 1A). The nucleotide sequence of the integrated fragments in p3'F-FatA is shown in Fig. 1B.

On the basis of the rapeseed genome size of 1,182 Mb per haploid genome [3], one haploid genome molecule corresponds to 1.22 pg of canola DNA, assuming that 965 Mb weighs 1 pg. Thus, according to the plasmid size, one plasmid molecule (2,903 bp) corresponds to 3.18 ag of plasmid DNA, considering that 965 Mb weighs 1 pg. For example, the mass of plasmid DNA containing 200,000 copes of the inserted sequence is as follows:

$$[3.18\times10^{-18}\ \text{g/copy}][200{,}000\ \text{copies}]=6.36\times10^{-13}\ \text{g}$$

Quantitative PCR

Real-time PCR assays with the TaqMan probe were performed using an ABI PRISM 7900 (Applied Biosystems), since the improved software of the 7900 System provides a

Table 1. Primer pairs and fluorogenic probes for quantitative PCR.

Name	Orientation	Sequences (5'→3')	Length	Amplicons size	Purpose
Fat-FR1	Sense	ATATACGGTTCGGGTGGAAG	20	111 bp	Quantitative
Fat-RR1	Antisense	CTCTGCATTCTCCGAGAATC	20		real-time
Fat-PR2	TaqMan probe	CAGACCTCTTGGCGTAGAAACATT	24		PCR
GT73-5	Sense	GACCATCATACTCATTGCTG	20	108 bp	
GT73-3	Antisense	GACACAAGCTTATACGAAGG	20		
GT73-P	TaqMan probe	TTCCCGGACATGAAGATCATCCTCCTTCC	29		

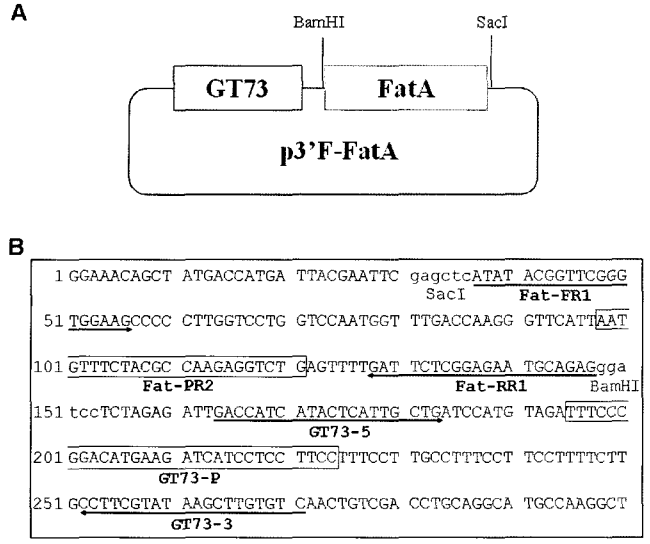

Fig. 1. Standard plasmid (p3'F-FatA) as reference molecule.
A. Schematic diagram of p3'F-FatA. FatA means canola endogenous gene and GT73 means 3'-flanking region inserted in GT73, with the same partial DNA sequences. **B.** Nucleotide sequence of integrated PCR amplicons in p3'-FatA. The arrows locate the primers with direction and the squared boxes indicate the TaqMan probes.

much more efficient analysis than the 7700 System. The PCR reaction mixture contained the following ingredients in final volumes of 25 μl: 50 ng samples of DNA, 0.5 μM primer pair, 0.2 μM probe, and 12.5 μl Universal Master Mix (Applied Biosystems). The real-time PCR reactions were run according to the following procedures: one cycle of 2 min at 50°C, 10 min at 95°C, 50 cycles of 30 s at 95°C, and 1 min at 59°C. Each sample was quantified in triplicate. The results were analyzed using a sequence detection system provided by the ABI 7900 software. The standard curves were calibrated using five concentrations of reference molecules from 20 to 200,000 copies per reaction. A no-template control (NTC) was prepared as the negative control for the analysis.

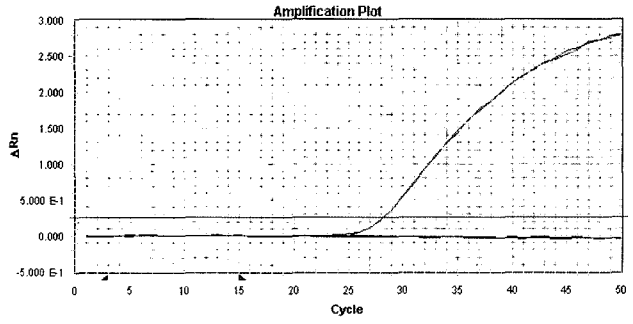

Fig. 2. Specificity analysis of canola endogenous *FatA* gene using quantitative PCR.
Amplification plot generated from 15 different plants species and canola with primer pair Fat-FR1/RR1 and probe FatPR2.

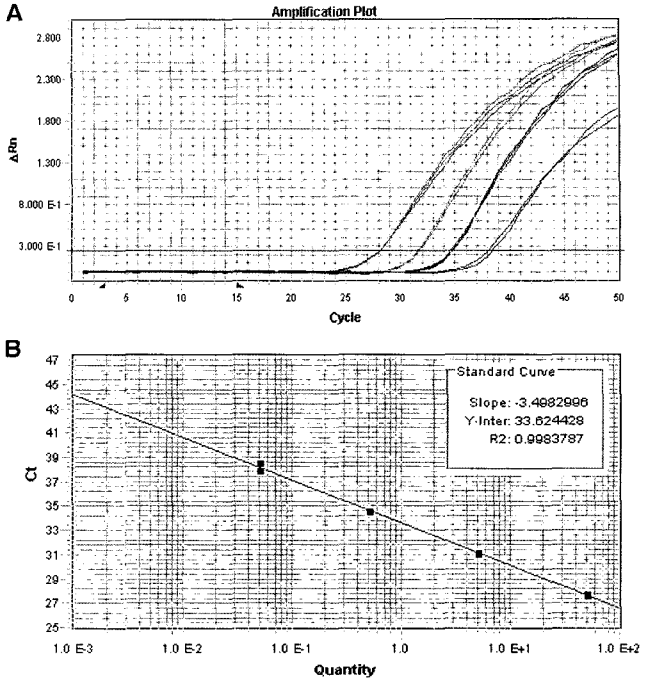

Fig. 3. Sensitivity detection of canola DNA fragments using quantitative PCR.
A. Amplification plot generated by serial dilution of canola DNA, ranging from 50 to 0.005 ng, with primer pair Fat-FR1/RR1 and FatPR-2 probe. **B.** Standard curve generated from amplification data given in **A.**

According to the principle of standard curve optimization, the best standard curves were used for the real-time PCR assays. The Ct values were used to determine the amount of total DNA using the endogenous *FatA* gene PCR system, and amount of transgenic DNA using the 3'-flanking gene PCR system based on the standard curves. The GMO contents were determined by the ratio of transgenic DNA copy numbers to total DNA copy numbers with the application of the coefficient values (Cv) [14].

RESULTS AND DISCUSSION

Specificity and Sensitivity of Specific Primer Pair of Canola Endogenous Gene, *FatA*, for Quantitative PCR Detection

A quantitative PCR system for GMOs depends on the copy number of endogenous reference genes, where the endogenous reference gene should be species-specific with a single copy number in the genome and exhibit a low heterogeneity among cultivars [15]. In the present study, the *acyl-ACP thioesterase* (FatA) gene (GenBank No. X87842) was selected as a canola endogenous gene for quantitative PCR detection. The specific primers and fluorogenic probe for this DNA sequence were designed for real-time quantitative PCR assays. As such, the primer pair Fat-FR1/RR1 and

Table 2. Reproducibility of Ct values using real-time PCR for five concentrations of genomic DNA from non-GM canola.

DNA amount (ng/reaction)	Ct value for reaction			Mean	SD[a]	CV(%)[b]
	1	2	3			
50	28.25	28.15	28.06	28.15	0.08	0.28
5	32.54	32.70	32.60	32.61	0.07	0.20
0.5	34.19	34.11	34.14	34.15	0.03	0.10
0.05	38.36	38.72	38.57	38.55	0.15	0.38
0.005	–	–	–	–	–	–

[a]Standard deviation.
[b]Coefficient of variation.

TaqMan probe Fat-PR2 were used to amplify the canola endogenous gene, *FatA*, in a quantitative PCR. To test the species specificity of the *FatA* gene in various species, a quantitative PCR was performed on 50 ng of template DNA from 15 different plant species. No amplification product was observed when using DNA samples from other species, such as rice, pepper, corn, soybean, tomato, perilla, sunflower, cucumber, buckwheat, cotton, mung beans, barley, wheat, chinese cabbage, and red-bean, as templates, demonstrating that the primer and probe set was specific for canola (Fig. 2).

To test the sensitivity of the real-time PCR system, real-time PCRs were carried out in triplicate based on three wells of five canola DNA dilutions, ranging from 50 ng to 5 pg, as templates using an ABI PRISM 7900 (Applied Biosystems). In the reproducibility tests, the PCR products could be amplified with as little as 50 pg of canola genomic DNA (Fig. 3). The Ct values ranged from 28.15 to 38.55, the standard deviation (SD) values ranged from 0.03 to 0.15, and the coefficient of variation (CV%) ranged from 0.10 to 0.38, as shown in Table 2, demonstrating that the CV values and SD values derived from these tests were relatively small and that the real-time PCR system worked stably and reliably.

Standard Plasmid for Real-Time PCR

The five standard plasmid concentrations were set at 2×10, 2×10^2, 2×10^3, 2×10^4, and 2×10^5 copies per reaction. The linearity of the standard curves for *FatA* and GT73 were confirmed by a quantitative PCR using the designed primer pairs, probes, and the standard plasmid, respectively. The square regression coefficients (R^2) were 0.997 and 0.998 for the *FatA* and event-specific PCR assay, respectively

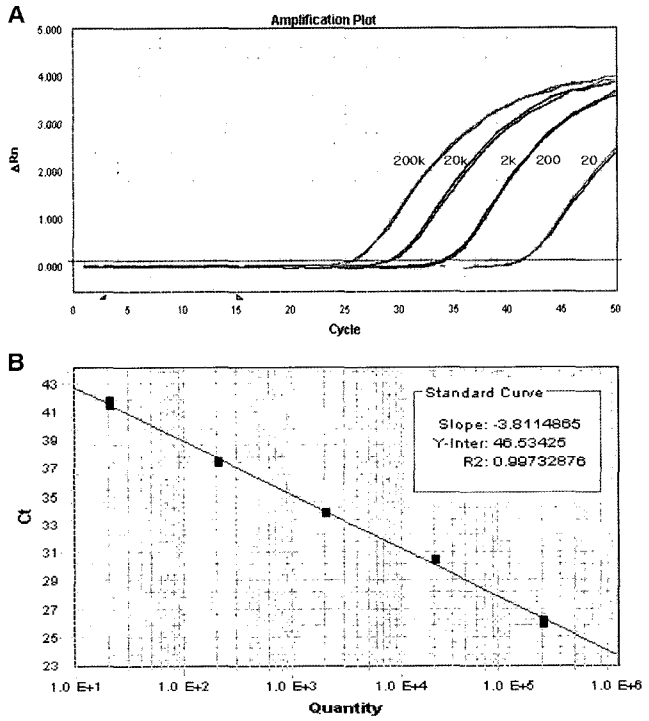

Fig. 4. Amplification plots and standard curve of quantitative real-time PCR assay for determining endogenous gene copy number in standard plasmid.
A. Amplification plots for canola endogenous *FatA* gene, established using 200,000, 20,000, 2,000, 200, and 20 copies of standard plasmid. **B.** Parameters of regression line through data points are indicated within the plot.

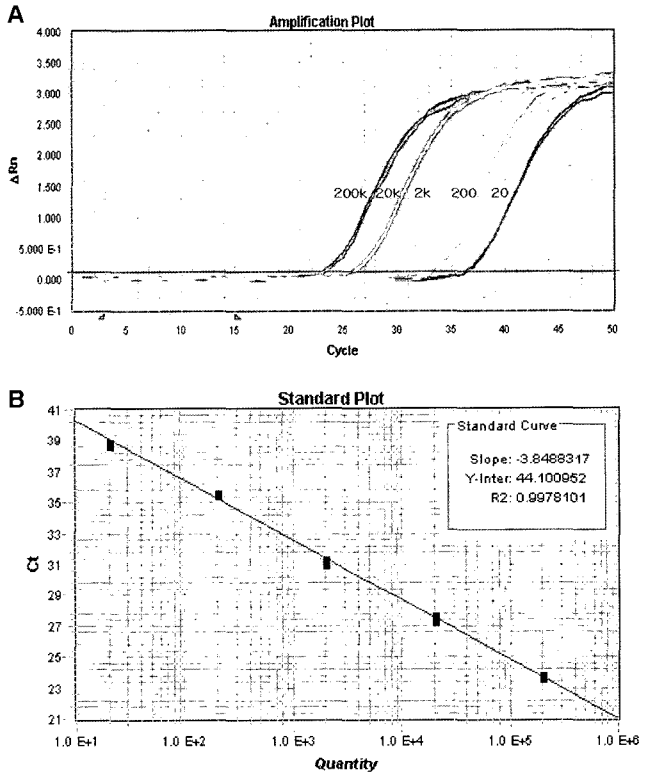

Fig. 5. Amplification plots and standard curve of quantitative real-time PCR assay for determining transgene copy number in standard plasmid.
A. Amplification plots for GM canola 3'-flanking gene, established using 200,000, 20,000, 2,000, 200, and 20 copies of standard plasmid. **B.** Parameters of regression line through data points are indicated within the plot.

Table 3. Repeatability of Ct measurements of replicate standards from 200,000 to 20 copies of standard plasmid DNA.

Target copies	Ct values			mean Ct	SD[a]	CV(%)[b]
	mean 1	mean 2	mean 3			
event specific assay						
200,000	25.52	25.41	25.33	25.42	0.08	0.31
20,000	28.38	28.25	28.32	28.32	0.05	0.19
2,000	31.85	31.70	32.02	31.86	0.13	0.40
200	35.67	35.60	35.56	35.61	0.05	0.13
20	39.48	39.79	39.57	39.61	0.13	0.33
FatA assay						
200,000	26.87	26.85	26.61	26.78	0.12	0.44
20,000	30.58	30.70	30.22	30.50	0.20	0.66
2,000	35.14	35.17	34.73	35.02	0.20	0.57
200	38.30	38.55	38.52	38.45	0.11	0.29
20	42.68	42.62	43.09	42.80	0.21	0.48

[a]Standard deviation.
[b]Coefficient of variation.

(Figs. 4 and 5). The fine linearity between the DNA quantities and fluorescence values (Ct) indicated that the assays were well-suited for quantitative measurements. The reproducibility of the Ct values was determined using the standard plasmid DNA dilutions in triplicate reactions (Table 3). The RT-PCR assay for the endogenous *FatA* gene detection gave mean Ct values from 26.78 to 42.80 cycles for each standard dilution, with standard deviation (SD) values of 0.11–0.21. Meanwhile, the event-specific PCR assay gene detected mean Ct values from 25.42 to 39.61 cycles for each standard dilution, with SD values of 0.05–0.13. The CV values ranged from 0.13 to 0.66. Thus, all the above results confirmed that p3'F-FatA was effective as a standard plasmid DNA for the quantification of GM-canola.

Accuracy and Precision of Quantitative PCR Method

Five samples with 10% to 0.1% of GT73 content were prepared by mixing the GT73 with non-GM canola genomic DNA. The quantitative results for the five samples were 10.03, 5.45, 3.80, 1.33, and 0.2%, respectively. The true value for the 0.1% sample was corrected to 0.2%, as below 20 copies were detected. The calculated mean, bias, and relative standard deviation (CV) at each mixing level are shown in Table 4. Repeated measurement of the standard DNA solution led to a CV for all methods within a range of 1.28–12.02%. In addition, the bias ranged from 0.31 to 98.37%. Similar ranges for the CV and bias have already been published in connection with other quantitative GMO detection systems [9, 10, 19], yet a low concentration of the target DNA leads to a higher variation among parallel runs than a high concentration.

In conclusion, the present study validated the *FatA* gene as an endogenous reference gene for canola. Event-specific quantitative real-time primers and probes were also developed for the detection of GT73, and a standard plasmid (p3'F-FatA) constructed as a reference molecule that can be easily amplified and is more economical and stable than seed powder. Therefore, this quantitative PCR analysis method could facilitate a sensitive labeling system within Korea GMO labeling regulations, with an acceptable level of accuracy and precision.

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Table 4. Accuracy statistics for quantitative method.

GM line	True value (%)	Coefficient value (Cv)	Accuracy				
			Trueness		SD[b]	Precision	
			Mean (%)	Bias[a] (%)		CV[c] (%)	Below 20 copies[d]
GM-Canola	10.0	0.78	10.03	0.31	0.13	1.28	0/3
	5.0		5.45	9.00	0.22	4.06	0/3
	3.0		3.80	26.68	0.44	11.69	0/3
	1.0		1.33	33.16	0.08	6.05	0/3
	0.1		0.20	98.37	0.02	12.02	1/3

[a]Bias=(mean value–true value)/true value×100.
[b]SD, standard deviation.
[c]CV, Relative standard deviation. The coefficient values are calculated by dividing the standard deviation by the mean value and are given as a percent.
[d]Below 20 copies, the number of experiments below 20 copies/total number of experiments.

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