Analytical Methods

Evaluation of real-time PCR detection methods for detecting rice products contaminated by rice genetically modified with a CpTI–KDEL–T-nos transgenic construct

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A B S T R A C T

Genetically modified (GM) rice (Oryza sativa) lines, such as insecticidal Kefeng and Kemingdao, have been developed and found unauthorised in processed rice products in many countries. Therefore, qualitative detection methods for the GM rice are required for the GM food regulation. A transgenic construct for expressing cowpea (Vigna unguiculata) trypsin inhibitor (CpTI) was detected in some imported processed rice products contaminated with Kemingdao. The 3′ terminal sequence of the identified transgenic construct for expression of CpTI included an endoplasmic reticulum retention signal coding sequence (KDEL) and nopaline synthase terminator (T-nos). The sequence was identical to that in a report on Kefeng. A novel construct-specific real-time polymerase chain reaction (PCR) detection method for detecting the junction region sequence between the CpTI–KDEL and T-nos was developed. The imported processed rice products were evaluated for the contamination of the GM rice using the developed construct-specific real-time PCR methods, and detection frequency was compared with five event-specific detection methods. The construct-specific detection methods detected the GM rice at higher frequency than the event-specific detection methods. Therefore, we propose that the construct-specific detection method is a beneficial tool for screening the contamination of GM rice lines, such as Kefeng, in processed rice products for the GM food regulation.

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1. Introduction

There has been great progress in food biotechnology, including genetic modification and transgenic crop breeding for food production. Genetically modified (GM) plants have been commercially grown for almost two decades (James, 2011). In some countries, the acceptance of these GM foods by consumers is still controversial, and concerns about their safety persist among the public. GM plants have been authorised for food by many countries based on their own criteria for safety assessment. In the European Union (EU), the authorisation and use of GM foods are stipulated by the provisions in regulations (EC) 1829/2003 and (EC) 1830/2003 (Regulation (EC) No. 1829/2003; Regulation (EC) No. 1830/2003). Japan has also announced a mandatory safety assessment of GM foods and processed foods containing GM plant ingredients. Since April 1, 2001, any unauthorised GM foods are prohibited from import or sale in Japan (Notification 79 of March 15). Therefore, the methods for qualitative detection of unauthorised GM foods are required for GM food regulation.

We have developed qualitative detection methods for several GM plants, such as New Leaf Plus and New Leaf Y potato (Solanum tuberosum) (Akiyama, Sugimoto, et al., 2002; Akiyama, Watanabe et al., 2002), Bt11, Event 176, MON810, T25, GA21 and CBH351 maize (Zea mays) (Akiyama et al., 2008; Matsuoka, Kuribara, Akiyama, et al., 2001; Matsuoka, Kuribara, Suefuji, et al., 2001; Matsuoka et al., 2000), FP967 flax (Linum usitatissimum) (Nakamura et al., 2010), and 55-1 and PRSV-YK papaya (Carica papaya) (Nakamura et al., 2011, 2013) using qualitative real-time polymerase chain reaction (PCR) methods. In the case of rice (Oryza sativa), unauthorised GM rice, herbicide-resistant Liberty-Link (LL) rice and insect-resistant Bacillus thuringiensis (Bt) rice (Shanyou63 and Kemingdao), have been found in some imported processed rice products in Japan (Akiyama et al., 2007, 2009). In EU, unauthorised GM rice has been found in foods imported to EU since around 2006...
(Decision 2008/289/EC; Reiting, Grohmann, & Mäde, 2010). Thus, the methods for detection of sequences characteristic of the unauthorised GM rice are required to monitor the foods for GM food regulating purposes.

In the present study, transgenic constructs for expression of insect-resistant cowpea (Vigna unguiculata) trypsin inhibitor (CpTI) and Bt toxin gene (cry1A(b)) were detected in the identical imported processed rice products. The construct harboured nucleotide sequences homologous to those in the GM rice line, Kefeng and Kemingdao. To detect the specific sequences of Kefeng and Kemingdao in processed rice products at high sensitivity, we compared the construct-specific real-time PCR detection methods with the event-specific real-time PCR detection methods targeting at the border sequence of the construct and the rice genome.

2. Materials and methods

2.1. Rice samples

Processed rice products imported to Japan that were suspected to be contaminated with GM rice on the basis of testing at a quarantine inspection center were obtained through the Ministry of Health, Labour, and Welfare of Japan. The non-GM rice variety Nipponbare (JP No. 9046) was purchased from NIAS Genebank at the National Institute of Agrobiological Sciences of Japan and used as a reference material.

2.2. Extraction and purification of genomic DNA

Rice samples were ground using an electric mill. DNA extraction and purification were carried out using the Nippon Gene GM quicker 2 kit (Nippon Gene, Toyama, Japan) according to the manufacturer’s instructions with the following modification: the ground samples (500 mg) were suspended in 2.1 ml of GE1 buffer, 60 µl of proteinase K (20 mg/ml), 6 µl of α-amylase (Nippon Gene), and 30 µl of RNase A (100 mg/ml) using a vortex mixer for 30 s and then heated at 65 °C for 30 min. A 255-µl aliquot of the GE2-K buffer was added to the mixture and mixed using a vortex mixer followed by standing on ice for 10 min. After centrifugation at 6000g at 4 °C for 15 min, the supernatant was transferred into a fresh tube and the mixture was centrifuged again at 13,000g at 4 °C for 5 min. To 1 ml of the supernatant placed in a new tube, 375 µl of both GE3 buffer and isopropanol were added, and the solution was then gently mixed by shaking 10–12 times. The mixed solution was applied onto a spin column included in the kit and centrifuged at 13,000g at 4 °C for 30 s. The eluate was discarded. This procedure was repeated until the entire solution was loaded. The spin column was washed with 650 µl of GW buffer by centrifugation at 13,000g at 4 °C for 1 min. The column was transferred to a new tube; 50 µl of TE buffer was added and allowed to stand for 3 min at room temperature. Finally, the column with the tube was centrifuged at 13,000g at 4 °C for 1 min, and the eluate was used as the DNA sample solution in the following experiments.

2.3. PCR

The PCR mixture (25 µl) in the tubes consisted of 2.5 µl 10 × PCR buffer II (Life Technologies, Carlsbad, CA), 0.16 mM dNTP, 1.5 mM MgCl2, 1.2 mM 5’ and 3’ primers and 0.8 units of AmpliTaq Gold DNA polymerase (Life Technologies). PCR was performed by preincubation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 30 s, annealing at 56 °C for 30 s, and extension at 72 °C for 30 s, then terminal elongation at 72 °C for 7 min using the GeneAmp PCR System 9700 (Life Technologies). For detection of the rice reference gene, sucrose phosphate synthase (SPS), amplifying primer sets (Spa-taq-1F and Spa-taq-1R) were used as described previously (Wang et al., 2010) (Table 1). After PCR, 5 µl of PCR mixture was electrophoresed at constant voltage (100 V) on a 2 % (w/v) agarose gel that had been supplemented with 0.5 µg/ml ethidium bromide in TAE [40 mmol/l Tris–HCl, 40 mmol/l acetic acid, and 1 mmol/l EDTA (pH 8.0)] buffer solution. A 100 bp DNA Ladder (Takara Bio Inc., Shiga, Japan) was used for size control of amplified fragments. The gel was photographed with a DIANA system (Raytest, Straubenhardt, Germany).

2.4. Analysis of genomic DNA sequence flanking the transgenic vector construct

To analyse the genomic DNA sequences flanking the T-DNA insertion sequence in the GM rice, the Straight Walk method (Tsuchiya, Kameya, & Nakamura, 2009) was used according to the kit manufacturer’s instructions (Bex, Tokyo, Japan). The gene-specific primer pair used for the first PCR was WP-1, 5'-CGG AGG CTG GCA GTC TTA TTA G-3', and SP-1 rice No. 2, 5'-CTT TCT CAT CAT CTT CAT CCC TGG AC-3'; the pair used for the second PCR was WP-2, 5'-ATG CCC CGG CTC TCT TTA GGG TTA CAC GAT TGC TT-3', and SP-2 rice No. 2, 5'-AAG CCG AGT GAC AGC AAT TCA ACC-3'.

2.5. DNA sequencing

The DNA fragments amplified were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany), and amplified fragments were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) according to the manufacturer’s instructions. The sequences of the insert were verified using pUC/M13 forward and reverse sequencing primers using an ABI PRISM 3700 DNA analyser (Life Technologies). The nucleotide sequences were analysed using Lasergene version 7.0 software (DNASTAR Inc., Madison, WI).

2.6. Real-time PCR and data analysis

Real-time PCR was performed using an ABI PRISM 7900 HT Sequence Detection System (Life Technologies). All reactions were run as quadruplicates in 96-well plates. PCR mixtures were placed in a 25-µl final volume containing 50 ng template DNA, 12.5 µl TaqMan Universal PCR Master Mix (Life Technologies), 750 nM each primer and 150 nM probe. The primer pair and probe were designed using Primer Express 2.0 software (Life Technologies). The reaction conditions included an initiation step for 10 min at 95 °C, followed by 50 cycles of 20 s at 95 °C and 1 min at 60 °C.

The baseline was set to cycles 3–15. The normalised reporter signal (ΔRn) threshold for plotting cycle threshold (Ct) values was set to 0.2 during exponential amplification. Reactions undergoing exponential amplification with a Ct value of <48, as judged by visual inspection of the respective ΔRn plots and multi-component plots, were scored as positive. If a Ct value could not be obtained, the reaction was scored as negative. Reactions with a Ct value of <48, but not showing exponential amplification, were scored as negative.

3. Results

3.1. Amplification of transgenic gene from processed rice products

To monitor the contamination of GM rice containing a transgenic construct for CpTI expression in the processed rice products, a primer set for qualitative PCR was designed for the CpTI (GenBank ID: EU088405.1) and nopaline synthase terminator
this study (Table 1). As shown in Fig. 1, a PCR product (454 bp) verse primer, sNOSR1, designed to these sequences were used in (Deng, Song, Xu, & Zhu, 2002). A forward primer, sSCKF1, and re-
CpTI transgenic construct to give insect resistance to GM rice non-GM rice samples (Fig. 1A). BLASTn (http://blast.ddbj.nig.ac.jp/
ence gene, PCR using primers specific for detection of a rice endogenous refer-
product (rice powder A) as a template. In contrast, the PCR using 
was obtained using genomic DNA purified from a processed rice 
PCR product identified a 
blastn?lang=ja) searches of the nucleotide sequence of the 454-bp 
2620
K. Nakamura et al. / Food Chemistry 141 (2013) 2618–2624
Oligonucleotidesa used in this study.

<table>
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<th>Test name</th>
<th>Name of oligonucleotide</th>
<th>Sequence (5′–3′)</th>
<th>Targetb</th>
<th>Amplicon size (bp)</th>
<th>Specificity</th>
<th>Source</th>
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<td>This study</td>
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<td></td>
<td>sNOSR1</td>
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<td>T-nos</td>
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<td>SPS-1F</td>
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</tr>
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<td>SPS-1R</td>
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<td>Bt rice (Kemingdao)</td>
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<td>3′-terminal</td>
<td>TGTTGATCATGGTTCTCAGTACA</td>
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<td>5′-terminal</td>
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<td>Wang, Zhu, Lai, and Fu (2011)</td>
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<td>N1_1</td>
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<td>Wang et al. (2012)</td>
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<td>N1_2</td>
<td>AAAACATTTAAGAATGAGGTTATC</td>
<td>Event specific sequence</td>
<td>187</td>
<td>Event specific sequence</td>
<td>Wang et al. (2012)</td>
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</table>

a Probes were designed with 5′-end labelled with fluorescent reporter 6-carboxyfluorescein (FAM) and 3′-end with fluorescent quencher 6-carboxytetramethylrhodamine (TAMRA) or with 3′-minor groove binder (MGB).

Table 1
Oligonucleotidesa used in this study.

(T-nos, GenBank ID: AJ007623.1). These genes have been used in a CpTI transgenic construct to give insect resistance to GM rice (Deng, Song, Xu, & Zhu, 2002). A forward primer, sSCKF1, and reverse primer, sNOSR1, designed to these sequences were used in this study (Table 1). As shown in Fig. 1, a PCR product (454 bp) was obtained using genomic DNA purified from a processed rice product (rice powder A) as a template. In contrast, the PCR using DNA purified from non-GM rice produced no visible band. The PCR using primers specific for detection of a rice endogenous reference gene, SPS, produced an 81-bp band for both rice powder A and non-GM rice samples (Fig. 1A). BLASTn (http://blast.ddbj.nig.ac.jp/blastn?lang=ja) searches of the nucleotide sequence of the 454-bp PCR product identified a CpTI fragment at the 5′ terminus and T-nos at the 3′ terminus (Fig. 1B). A total of 229 bp sequences encoding the C-terminal part of the CpTI and 166 bp sequences encoding T-nos were identical to the sequences deposited in GenBank (GenBank IDs: EU088405.1 [CpTI] and AJ007623.1 [T-nos]). This transgenic construct was designed to express CpTI with the four amino-acid endoplasmic reticulum retention signal sequence, Lys-Asp-Glu-Leu (KDEL), at the C-terminus. A stop codon (TAG) has been inserted at the 5′ terminus of the T-nos. The sequence in Fig. 1B also showed a point mutation inserted at adenine in place of thymine to code for the Lys of the KDEL. The presence of these identified sequence segments (CpTI–KDEL, stop codon and T-nos) suggests that the processed rice product rice powder A contained unauthorised GM rice.

To investigate the 5′ terminal upstream sequence of the transgenic construct, including the promoter region and the integration site in the rice genome, from the identified CpTI–KDEL, the Straight Walk method (Tsuchiya et al., 2009) was performed. We found that the processed food sample with rice powder A had an omega enhancer site upstream of the CpTI, which was inserted in the rice genome DNA at a site equivalent to chromosomes 3 and 9 of japonica variety Nipponbare (data not shown), although we did not detect the complete T-DNA insertion sequence including the promoter at the 5′ terminus of the CpTI-coding sequence.

3.2. Development of a construct-specific method for detection of GM rice expressing CpTI–KDEL using real-time PCR

To develop a sensitive and specific detection method for CpTI transgenic GM rice in processed rice products, a set of amplification primers and probe for real-time PCR was designed to hybridise to the sequence specific to the transgenic construct identified in
rice powder A (Fig. 2). The primer pair and probe, CpTI-2F/NOS-1R and KDEL-P, respectively, were designed at the border sequence between the CpTI-coding sequence and T-nos. The probe was designed to hybridise at the KDEL-coding sequence. The complete nucleotide sequences of the primers and probe are listed in Table 1. To confirm the specificity of real-time PCR detection test using these primers and probe set (CpTI–KDEL–T-nos), DNA purified from rice powder A was used as a template. An amplification curve generated during real-time PCR detection was obtained using 50 ng DNA template obtained from rice powder A with an average Ct value of 39.32 ± 0.18, whereas no amplified product was observed with non-GM rice (Table 2). Using these DNA templates, Ct values obtained for the endogenous rice SPS sequence gave 23.55 ± 0.04 and 23.46 ± 0.13 for rice powder A and non-GM rice, respectively.

To investigate whether any additional transgenic genes were present in genomic DNA purified from rice powder A, construct-specific real-time PCR detection methods to detect Shanyou63 (primer pair and probe: T51-SF/OsNOS-R2 and NGMr-Taq) and Kemingdao (primer pair and probe: T51-SF/OsNOS-R2 and NGMr-Taq) were used (Table 1). As we previously reported (Akiyama et al., 2007), the GM rice lines Shanyou63 and Kemingdao have been found in some processed rice products, such as vermicelli imported from China to Japan. Shanyou63 has a transgenic construct for expressing Bt toxin of the fusion type Cry1A(b)/Cry1A(c) under an rice Act1 promoter and Kemingdao has a transgenic construct for expressing Cry1A(b) under a maize ubiquitin promoter (Akiyama et al., 2007; Reiting et al., 2010). The real-time
Table 2

<table>
<thead>
<tr>
<th>Sample</th>
<th>Method name</th>
<th>Ct valuesb</th>
<th>Shanyou63</th>
<th>Kemingdao1</th>
<th>Kefeng6–1</th>
<th>Kefeng6–2</th>
<th>Kefeng6–3</th>
<th>Kefeng8</th>
<th>Non-GM</th>
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<tr>
<td>Rice powder A</td>
<td>23.55 ± 0.04 (4/4)</td>
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<td>Rice powder B</td>
<td>23.85 ± 0.03 (4/4)</td>
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<tr>
<td>Rice powder C</td>
<td>23.64 ± 0.04 (4/4)</td>
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<td>Rice powder D</td>
<td>23.44 ± 0.01 (4/4)</td>
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<tr>
<td>Non-GM</td>
<td>23.46 ± 0.13 (4/4)</td>
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</table>

* Ct values were obtained at the threshold value of 0.2. Values represent means ± SD of data of four test wells. ND: amplification signal is not detected. Numbers in parentheses indicate number of the positive wells per test resulting in amplification signal detected with Ct value less than 48 at threshold value of 0.2.

**Square brackets indicate the nucleotide sequence specificity.

3.3. Real-time PCR detection of GM rice expressing *CpTI–KDEL*

In 2010, a notification through the EU’s Rapid Alert System for Food and Feed (RASFF) announced that a trace of the unauthorised GM line Kefeng6, which harbours transgenic constructs for expressing Cry1A(c) and *CpTI*, had been detected (Reiting et al., 2010). To test whether the processed rice products contained Kefeng6 as notified by the EU, the previously used event-specific real-time PCR methods for GM rice lines Kefeng6, Kefeng6-1 (primer pair and probe: QK6-5F/QK6-5R and QK6-5P), Kefeng6-2 (primer pair and probe: Y6D-F/Y6D-R and Y6-P) and Kefeng6-3 (primer pair and probe: Kef6 forward/Kef6 reverse and Kef6 hydrolysis probe), were used (Guertler, Huber, Pecoraro, & Busch, 2012; Su, Xie, Wang, & Peng, 2011; Wang & Johnston, 2007). In addition, an event-specific detection method for another Kefeng line, Kefeng8, using primers K8-R2 and N1-1 and probe K8-P, was used (Wang, Zhu, Lai, & Fu, 2012). Four wells (each well containing 50 ng template DNA purified from rice powder) in a 96-well plate were tested using these event-specific real-time PCR detection methods (Table 2). Of the four processed rice products (rice powder A–D), one out of four wells (25% (1/4) of four repetitive tests) of rice powder A sample tested positive for both Kefeng6-2 and Kefeng6-3 at Ct values of 41.66 and 39.91, respectively, two out of four wells (50% (2/4) of four repetitive tests) of rice powder B tested positive for Kefeng6-1 at Ct values of 40.50/39.18 and one out of four wells (25% (1/4) of four repetitive tests) tested positive for Kefeng6-3 at Ct value of 38.80, and two out of four wells (50% (2/4) of four repetitive tests) of rice powder D were detected as positive for Kefeng6-1 at Ct values of 38.88/40.00. These results indicated that rice powder A, B and D contained Kefeng6 (Table 2). All samples tested negative using the event-specific detection method for Kefeng8. All event-specific detection methods failed (0% (0/4) of four repetitive tests) to detect Kefeng lines in rice powder C.

4. Discussion

We detected genomic DNA containing transgenic cassettes for expression of *CpTI–KDEL* and *cry1A(b)* in four imported processed rice products. Among processed rice products we monitored the contamination of GM rice, *CpTI–KDEL–T-nos* sequence was detected in 100% (4/4) of four repetitive tests, and GM rice line Kefeng6 event-specific sequences were detected in <50% (2/4) of four repetitive tests. Furthermore, GM rice line Kemingdao construct-specific sequence at junction region between *cry1A(b)* and T-nos was detected in 100% (4/4) of four repetitive tests. The construct-specific detection methods (*CpTI–KDEL–T-nos* and
Kemingdao were beneficial tool for screening GM rice contamination in processed rice products, whereas the event-specific detection methods were useful for identification of GM rice lines.

There were at least three possibilities for the origin of the transgenic construct in the GM rice detected using the construct-specific detection methods: (1) a GM rice line expressing CptI and Cry1A(b) developed using a single transgenic construct; (2) a two-line hybrid GM rice expressing CptI and Cry1A(b); (3) a mixture of two independent GM rice lines, one expressing CptI and another expressing Cry1A(b). GM rice lines that express neither CptI nor Cry1A(b) are presently approved for food and feed use in many countries, such as EU and Japan. In this study, our new construct-specific detection method for CptI–KDEL–T–nos identified the presence of a CptI transgenic cassette in DNA purified from processed rice products, a contaminant that the event-specific detection methods may fail to detect. These results indicate that the construct-specific detection methods targeting the transgenic construct sequences might be appropriate for identification of GM rice line. However, we cannot estimate the detection limit of these real-time PCR methods for the GM rice since we do not have any authentic reference materials for the CptI and Cry1A transgenic rice lines. Further studies are required to examine the feasibility of detecting lower levels of contamination by GM rice lines using reference materials and to extend the applications of the method to more complex processed food products.

5. Conclusions

A transgenic construct for expressing CptI and Cry1A(b) was detected in processed rice products using construct-specific real-time PCR. The construct-specific detection methods targeting the expression cassette sequences were beneficial tool for monitoring processed rice products for detection of a trace contamination originating from unauthorised GM rice lines, such as Kefeng and Kemingdao.

Acknowledgement

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References


Akiyama, H., Sasaki, N., Sakata, K., Ohmori, K., Toyota, A., Kikuchi, Y., et al. (2007). Indicated detection of two unapproved transgenic rice lines contaminating A transgenic construct for expressing CptI and Cry1A(c) expressing GM rice line, such as Shanyou63, was detected using primers T51-SF and OsNOS-R2 and probe GM63-Taq. DNA from a Cry1A(b) expressing GM rice line, Kemingdao, was detected using primers T51-SF and OsNOS-R2 and probe NGMr-Taq, or primers KM2_for and KM1_rev and probe KM_p. In this study, Kemingdao-positive rice was further checked for detection of a CptI transgenic construct using primers CptI-2F and NOS-1R and probe KDEL-P. Event-specific detection methods (Kefeng6-1, Kefeng6-2, Kefeng6-3 and Kefeng8) were used to identify contamination from GM rice lines Kefeng6 and Kefeng8. Square brackets indicate the specificity of tests; parentheses are the test result (+, positive), which is indicated by thick lines, for identification of GM rice lines.


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