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Full Length Research Paper

Species-specific detection of *Dickeya* sp. (*Pectobacterium chrysanthemi*) in infected banana tissues, soil and water

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***Dickeya* sp. (*Pectobacterium chrysanthemi*) is a bacterial pathogen that can cause soft rot disease on banana pseudostem tissue. A species-specific polymerase chain reaction (PCR) assay was developed for rapid and sensitive detection of the pathogenic bacteria in diseased plant tissues, soil and artificially infested irrigation water. Based on differences in internal transcribed spacer (ITS) sequences of *Dickeya* sp. (*P. chrysanthemi*) and other bacteria, a pair of species-specific primers was synthesized. Specificity was tested against eight bacterial organisms associated with banana. The LF/LR primers amplified only a single PCR band of 171 bp from *Dickeya* sp. (*P. chrysanthemi*). The detection sensitivity was determined to be 0.44 fg for pure genomic DNA per 25 µl reaction volume. The results suggest that the assay detected the pathogen more rapidly and accurately than standard isolation methods. The PCR-based methods developed here could simplify both plant disease diagnosis and pathogen monitoring, as well as guide plant disease management.**

Key words: Soft rot, banana, detection, polymerase chain reaction (PCR).

INTRODUCTION

Dickeya sp. (*Pectobacterium chrysanthemi*) is a plant-pathogenic enterobacterium responsible for soft rot disease in a wide range of species (Lee and Yu, 2006). The gram-negative bacterium, important pathogen, first identified on banana in 2009 at Panyu, Guangzhou, China (Lin et al., 2010), was accountable for significantly economic losses to banana production. This pathogen is survives in stagnant irrigation water and soil, often in association with decaying crop residues. Usually contamnation or infection is a subclinical level; such latent infections cannot be detected by visual inspection.

Polymerase chain reaction (PCR) techniques offer advantages over traditional methods of detection and diagnosis. The practice of diagnosing plant pathogens using PCR has previously been described (Henson and French, 1993; Lévesque, 2001; McCartney et al., 2003; Atkins and Clark, 2004). Plant diseases can be controlled most effectively if diagnostics is introduced at an early stage of disease development. Information resulting from molecular diagnostics could be used to make more rational decisions about the choice and use of proper agrochemicals at optimal application times. Accurate and early diagnosis of a specific pathogen is greatly important in a crop protection system or could be useful in selection within the breeding programs. Standard diagnostic methods, such as visual assessment of symptoms, as well as microscopic or cultural methods, often have limited potential. External symptoms can be masked, especially if pathogens occur simultaneously. The microscopic method evaluation mostly requires specialized training, experience and the taxonomical knowledge of an investigator and cultural techniques can also be time-consuming (Matusinsky et al., 2010). Following the appearance of the PCR technology, because of sensitivity, accuracy and relatively rapid results, molecular detection method has been widely used for pathogen diagnoses.

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Abbreviations: PCR, Polymerase chain reaction; **ITS**, internal transcribed spacer.

Figure 1. Symptoms appearing on healthy banana leaves after inoculation treatments. A, Leaves injected with *Dickeya* sp. (*Pectobacterium chrysanthemi*) bacterial suspension; **B**, leaves injected with distilled water, designed as control.

In this study, PCR primers derived from 16S-23S rDNA internal transcribed spacer (ITS) sequences were designed for the specific detection of *Dickeya* sp. (*P. chrysanthemi*) (Gurtler and Stanisich, 1996; Kwon et al., 2000). The specificity and sensitivity of the reaction were tested and the PCR protocols were used to detect diseased plant tissues, irrigation water, and infested soil samples collected in the field.

MATERIALS AND METHODS

Isolates

In 2009, *Dickeya* sp. (*P. chrysanthemi*) strains were isolated from naturally diseased banana pseudostem tissue at the growth stage showing soft rot symptoms in various cultivation areas of Gangdong province, China. Individual colonies isolated were further assayed by injecting 20 µl 10^9C FU/ml bacterial suspension into the healthy banana leaves to test pathogenicity (Lee and Yu, 2006) (Figure 1).

Bacterial strains and DNA extraction

The bacterial strains used in this study are listed in Table 1. The reference strains were grown in 30 ml of nutrient broth for 24 to 48 h on an orbital shaker at 35°C. Cultures were harvested in late log phase and genomic DNA was extracted using a commercial DNA purification kit (QIAGEN, France). DNA of infected and non-infected banana pseudostem tissue was extracted according to Tooley et al. (1997) using 1 g of tissue samples that were previously ground in liquid nitrogen. Soil samples DNA was extracted directly according to the method of Li (Li and Hartman, 2003). A TENP extraction buffer (containing 50 mm Tris, pH 8.0; 20 mm ethylene diamine tetraacetic (EDTA) disodium salt pH 8.0, 100 mm NaCl and 1% polyvinylpolypyrrolidone) was added to the lyophilized soil suspended in 0.5 ml 0.4% dry milk powder solution. After vortexing, samples were incubated at 65°C for 30 min, and then 10 ml of 20% (w/v) sodium dodecyl sulfate (SDS) was added. The samples were incubated at 65°C for another 30 min, then samples were centrifuged at 18 000 *g* for 10 min. The supernatant was transferred to a new centrifuge tube, and half volume of 7.5 m ammonium acetate and two volumes of 100% ethanol at -20°C for 2 h were added. DNA was pelleted, rinsed with 70% ethanol, dried, dissolved in sterile water and stored at -20°C. DNA yield and purity were determined spectrophotometrically.

PCR amplification conditions

PCRs were carried out in a GeneAmp PCR systems 9700 thermocycler (Applied Biosystems, Courtaboeuf, France) in 25 µl containing 1× Mg^{2+}-free PCR buffer (Gibco-BRL, Burlington, Ontario, Canada), 2.0 mM $MgCl_2$, 0.16 mM dNTPs each, 0.4 µM primer each, 2.5 units of Taq DNA polymerase (Gibco/BRL), and 1 µl of genomic DNA (10-15 ng/µl) with the following thermal profile: 94°C for 5 min followed by 35 cycles of 94°C for 30 s, 53°C for 20 s, and 72°C for 30s , and a final extension at 72°C for 10 min.

16S-23S rDNA gene ITS PCR amplification

The *Dickeya* sp. (*P. chrysanthemi*) 16S-23S rDNA ITS region was amplified by PCR. The PCR product with the expected size of approximately 440 bp was obtained with universal primers (primer1: 5'-GAAGTCGTAACAAGG-3'; primer2: 5'-CAAGGCATCCACCGT-3') for bacterial ITS amplification described earlier by Jensen et al. (1993). The purified PCR products were cloned into the plasmid vector pMD20-T (TaKaRa), and positive clones were sequenced in Invitrogen Company. The sequence was submitted to Genbank.

Specific primers design

Specific primers for *Dickeya* sp. (*P. chrysanthemi*) were designed by the comparison of the ITS of 18 different *Erwinia* sequences from GenBank (Table 2). The pair of primers were designed using Primer3Plus software (Untergasser et al., 2007), and they were analyzed using the Net-Primer (Biosoft International) software (NetPrimer) which identifies regions of likely secondary structure (hairpins) and potential primer dimer formation and assigns values for melting temperature (Tm). The specificities of the primers were checked using FASTA and basic local alignment search tool (BLAST) searches of the GenBank databases.

Table 1. Bacterial isolates used to screen the specifictity of the primers.

Species	Host	Origin	Amplification with primers LF/LR
Dickeya sp. (*Pectobacterium chrysanthemi*)	Banana	Guangdong	+
Pectobacterium chrysanthemi	Unknown	ATCC11663	+
Erwinia amylovora	Unknown	Zhejiang	-
Erwinia carotovora subsp. *carotovora*	Carrot	Yunnan	-
Xanthomonas campestris pv . musacearum	Banana	CGMCC	-
Ralstonia solanacearum race 2	Banana	CGMCC	-
Pantoea stewartii subsp. *Stewartii*	Corn	Shandong	-
Xanthomonas oryze pv.oryze	Rice	Guangdong	-
Curtobacterium flaccumfaciens pv. flaccumfaciens	Unknown	CGMCC	-
Pseudomonas syringae pv. syringae	Unknown	CGMCC	-

+, 171bp product amplified by primers LF/LR; **-**, no amplified products; CGMCC, China general microbiological culture collection; ATCC, American type culture collection.

Table 2. GeneBank accession numbers and sequences compared to develop species-specific primers LF/LR for *Dickeya* sp. (*Pectobacterium chrysanthemi*).

Accession number	Species	Accession number	Species
GQ181062	*Erwinia amylovora*	EF427910.1	*Erwinia pyrifoliae*
HM130661.1	*Pectobacterium carotovorum*	EF427911.1	*Erwinia pyrifoliae*
HM130662.1	*Pectobacterium carotovorum*	EF427912.1	*Erwinia pyrifoliae*
HM130663.1	*Pectobacterium carotovorum*	EF427913.1	*Erwinia pyrifoliae*
HM130664.1	*Pectobacterium carotovorum*	EF427914.1	*Erwinia pyrifoliae*
EF427906.1	*Erwinia pyrifoliae*	EF422407.1	*Erwinia pyrifoliae*
EF427907.1	*Erwinia pyrifoliae*	AF232681.1	*Erwinia chrysanthemi*
EF427908.1	*Erwinia pyrifoliae*	AF232682.1	*Erwinia chrysanthemi*
EF427909.1	*Erwinia pyrifoliae*	AF232683.1	*Erwinia chrysanthemi*

Test of primers specificity and sensitivity

The designed primer pairs were tested for their specificity against the DNA of *Dickeya* sp. (*P. chrysanthemi*) isolated on diseased banana pseudostem tissue and other bacterial pathogens associated with disease of crops. Furthermore, the potential for detection was tested in banana pseudostem tissue infected with *Dickeya* sp. (*P. chrysanthemi*) compared with non-infected ones. The sensitivity of diagnostic PCR was determined for *Dickeya* sp. (*P. chrysanthemi*) by testing a 10-fold dilution series of known DNA concentration. DNA extracts were initially tested at concentrations on the order of 44 ng/µl; the concentration was decreased until no products could be observed by agarose gels. All reactions were repeated at least three times.

RESULTS

16S-23S rDNA gene ITS sequence analysis

16S–23S rDNA coding spacer regions of the *Dickeya* sp. (*P. chrysanthemi*) were amplified using universal primers (primer1/2) for bacterial ITS amplification (Jensen et al., 1993) and products were cloned and sequenced (Genbank no:HQ882841). The 440 bp length sequence was identical with sequence from *Dickeya* sp. (*P. chrysanthemi*) strain ATCC 11663 (Type strain, GenBank Accession no. AF232681.1) submitted previously, which further enhance authenticity of our study.

Specificity and sensitivity of PCR amplification

Specific primers for *Dickeya* sp. (*P. chrysanthemi*) (LF 5'-TTCGTCTAGAGGCCCAGGAC-3' and LR 5'-TCAGCTTGTTCCGGATTGTT-3') were designed. In the essential specificity test, the primer set LF/LR was able to amplify a unique DNA fragment of approximately 171 bp (Figure 2) from all *Dickeya* sp. (*P. chrysanthemi*) isolates tested from naturally diseased banana pseudostem tissue.

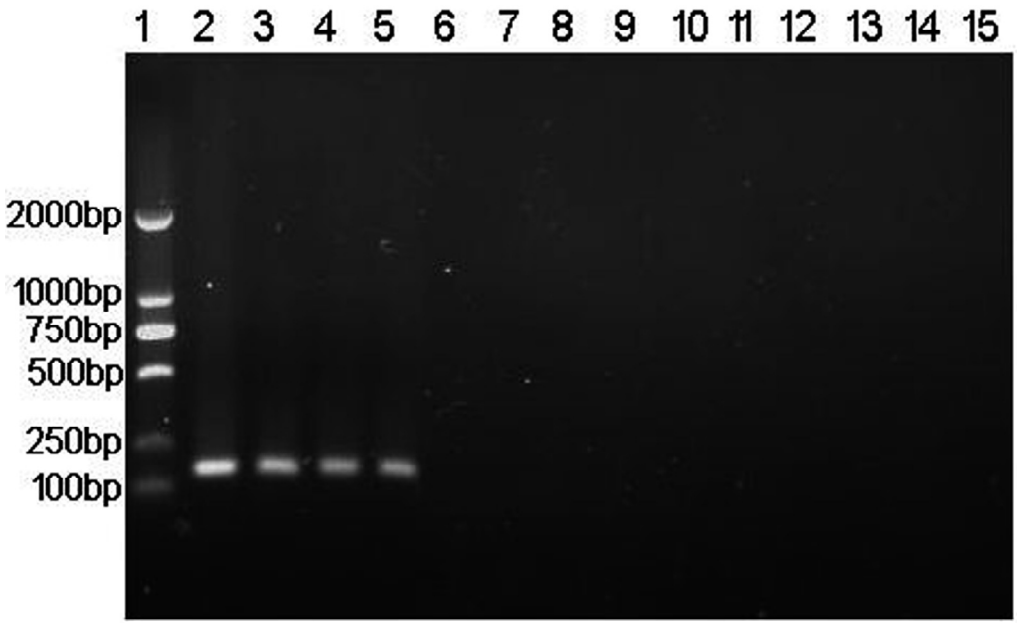

Figure 2. Agarose gel electrophoresis of PCR-amplified products using the specific primers LF/LR. Lane 1, 2000bp DNA marker; lane 2, *P. chrysanthemi* (ATCC11663); lanes 3 to 4, *Dickeya* sp. (*Pectobacterium chrysanthemi*) isolates; lane 5, infected banana tissues; lane 6, *Erwinia amylovora*; lane 7, *Erwinia carotovora* subsp. *carotovora*; lane 8, *Xanthomonas campestris pv . musacearum*; lane 9, *Ralstonia solanacearum race* 2; lane 10, *Pantoea stewartii* subsp. *Stewartii*; lane 11, *Xanthomonas oryze pv.oryze*; lane 12, *Curtobacterium flaccumfaciens pv. flaccumfaciens*; lane 13; *Pseudomonas syringae pv. syringae*; lanes 14, non-infected banana tissues; lane 15, ddH_2O.

However, the other reference strains tested showed no amplification product. The primer set was therefore used for further analysis. The primers LF/LR were used to examine infected and non-infected plant materials and one sharp band (171 bp) was amplified in infected banana tissues and non-infected not.

The sensitivity of diagnostic PCR was assessed by determining the minimum amount of *Dickeya* sp. (*P. chrysanthemi*) DNA. Amplification reactions were conducted with 10-fold decreasing amounts of genomic DNA of *Dickeya* sp. (*P. chrysanthemi*). In a 25 µl reaction volume assay, primers LF/LR were able to detect 0.44 fg of pure genomic DNA (Figure 3A).

Detection in plant tissues

The PCR assay with the pair of species-specific primers LF/LR detected *Dickeya* sp. (*P. chrysanthemi*) in naturally infected plant tissues from Hainan, Guangdong, Guangxi, and Fujian. This PCR method succeeded in amplifying the expected 171 bp product using DNA samples extracted from all soft-rot infected banana samples (Figure 3B). In contrast, no PCR product was amplified from healthy banana tissues. This indicates that the DNA amplified in PCR assays by primer sets LF/LR was derived from *Dickeya* sp. (*P. chrysanthemi*).

Detection in infested field soil and artificially infested irrigation water

DNA extracted from field soil samples collected from 12 infested banana fields in Guangdong and twice-autoclaved field soil sample (healthy banana fields as negative control) was subjected to PCR using primers LF/LR. The DNA samples extracted from 1 g soil were suspended in 20 µl ultrapure water used for PCR amplification. 10 of the 12 samples provided 171 bp PCR products amplified by LF/LR (Figure 3C), while PCR with the pair of primers yielded the 171 bp band in irrigation water artificially infested with *Dickeya* sp. (*P. chrysanthemi*) 10^9 CFU/ml bacterial suspension. The detection limit of PCR template was 10^3 CFU/ml in 25 µl volumes reaction (Figure 3D), indicating that the method used in this study can sensitively detect the pathogen in water.

DISCUSSION

Molecular technologies have become some of the most important tools for identifying and detecting plant pathogens (Matusinsky et al., 2010). The rapid and reliable detection of pathogens is important for disease management, plant breeding, and epidemiological studies. In this study, our objective was to develop a rapid, sensitive, and effective

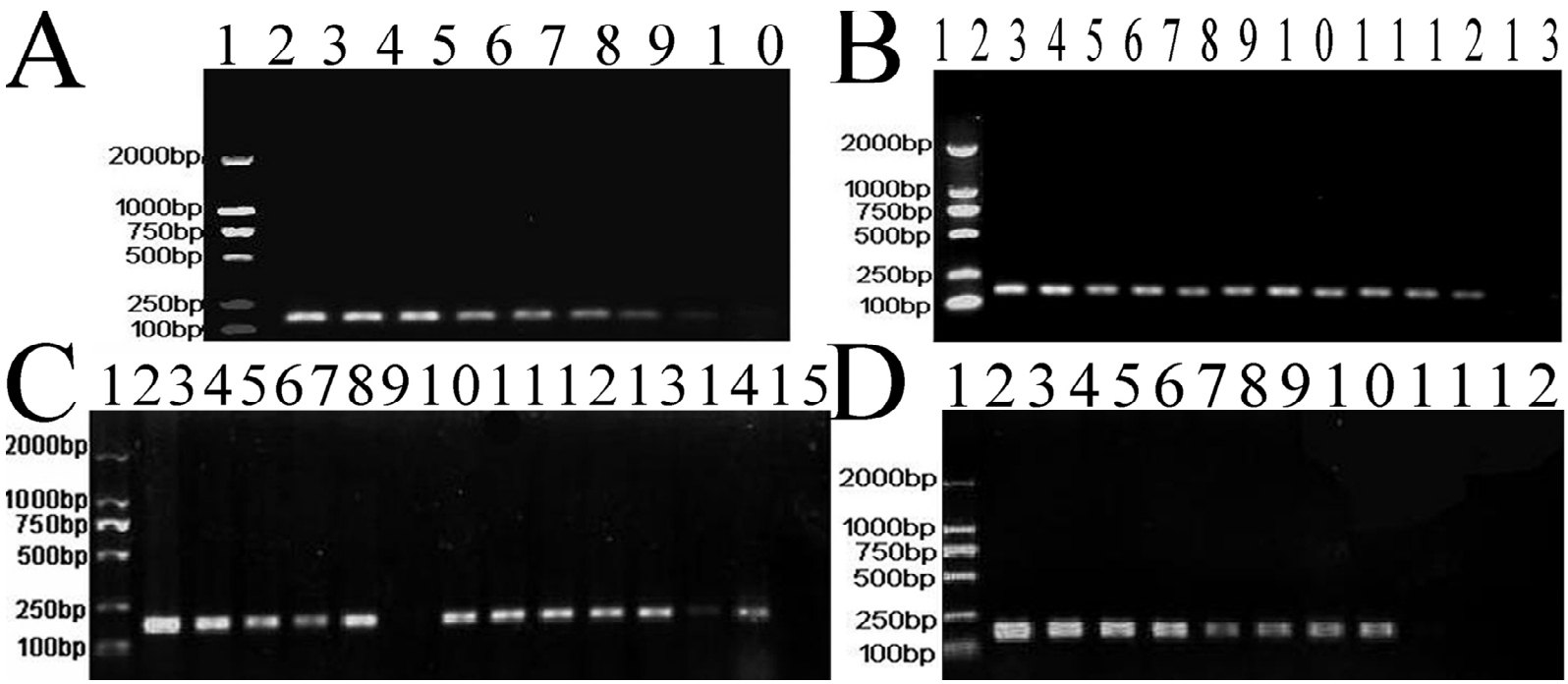

Figure 3. A, Sensitivity of PCR for detection of *Dickeya* sp. (*Pectobacterium chrysanthemi*). Lane 1, 2000bp DNA marker; lanes 2 to 9, amplified products using DNA at concentrations of 440 pg, 44 pg, 4.4 pg, 440 fg, 44 fg, 4.4 fg, 440 ag and 44 ag in a 25 µl PCR reaction; lane 10, ddH_2O. The same results were obtained in 3 replicates; **B**, PCR amplification of DNA extracted from diseased plants; lane 1, 2000bp DNA marker; lane 2, DNA from *Dickeya* sp. (*Pectobacterium chrysanthemi*); lanes 3 to12, amplified products using DNA from diseased banana tissues; lane 13, DNA from healthy banana tissues; C, products amplified from DNA extracted from soil samples; lane 1, 2000bp DNA ladder marker; lane 2, DNA from *Dickeya* sp. (*Pectobacterium chrysanthemi*); lanes 3 to 14, amplified products using DNA from infested soil samples; lane 15, negative control; D, products amplified from different concentrations of irrigation water infested with *Dickeya* sp. (*P. chrysanthemi*) bacterial suspension; lane 1, 2000bp marker; lane 2, DNA from *Dickeya* sp. (*Pectobacterium chrysanthemi*); lanes 3-11, Amplified products using infesed irrigation water at concentrations of 10^9 CFU/ml, 10^8 CFU/ml, 10^7 CFU/ml, 10^6 CFU/ml, 10^5 CFU/ml, 10^4 CFU/ml, 10^3 CFU/ml, 10^2 CFU/ml, 10 CFU/ml; lane 12, ddH_2O.

molecular method using species-specific PCR to identify and detect *Dickeya* sp. (*P. chrysanthemi*) which cause an odorous soft rot of the center of banana rhizome (Lin et al., 2010).

PCR-based assays applied to detecting plant pathogens have been described (Henson and French, 1993; Tooley et al., 1997; Cullen et al., 2002; Mercado-Blanco et al., 2003) as well as to monitor microorganisms in soils (Cullen et al., 2001; Lees et al., 2002; Carnegie et al., 2003). Most of the primers were designed for the rDNA region, and particularly the ITS region (Nazar et al., 1991; Ma and Michailides, 2002; Zhao et al., 2007) or other conserved genes, for example, b-tubulin (Hirsch et al., 2000), elongation factors (Filion et al., 2003; Li and Hartman, 2003), mitochondrial DNA (mtDNA) (Frederick et al., 2000; Tooley et al., 2006) and so on. The same to the previous study (Nazar et al., 1991; Ma and Michailides, 2002; Zhao et al., 2007), we designed specific primers from *Dickeya* sp. (*P. chrysanthemi*) 16S-23S rDNA ITS sequence.

The rDNA genes are essential for the survival of all organisms and are highly conserved in the

bacterial and other kingdoms. In addition, sequence information of 16S-23S spacer region suggests that considerable variation can occur between species in both the length and the sequence of this region. Therefore spacer variations between strains, species and genera may be used for identification and typing purposes (Gurtler and Stanisich, 1996). In our research, 16S–23S rDNA coding spacer regions of *Dickeya* sp. (*P. chrysanthemi*) were cloned and sequenced, and specific primers (LF/LR) were designed to amplify PCR products of 171 bp.

Some diseases have similar symptoms in early stages and infection is a subclinical level. Besides, agricultural field soil is a complex ecosystem with a diverse microbial community (Torsvik and Øvreås, 2002). There are many hundreds of different species of microorganism including fungi, bacteria and nematodes that are found in field soil. Thus, it is difficult and time-consuming to detect the presence and distinguish *Dickeya* sp. (*P. chrysanthemi*) from the other microbes in plant tissues and soil with standard diagnostic methods. However, the PCR detection method reported here can provide a definitive diagnosis of *Dickeya* sp. (*P. chrysanthemi*) in infected banana tissues, soils and water within hours. Moreover, this method is very easy to use and requires minimal training. The specific primer set LF/LR could not only distinguish *Dickeya* sp. (*P. chrysanthemi*) from other pathogens but could also accurately diagnose and survey the pathogen from diverse areas of China. The primers were sensitive enough to accurately amplify as little as 0.44 fg of diluted pure genomic DNA and 10^3 CFU/ml irrigation water artificially inoculated with *Dickeya* sp. (*P. chrysanthemi*) suspension in 25 µl reaction volume assay (Figures 3A and 3D). This level of detection limit may be of particular importance where it is desirable to detect the pathogen before disease symptoms have developed and most of plants are infected.

This PCR-based detection can be used together with standard diagnostic methods focused on plant pathology. If needed, results of analyses will be available in a few hours. Thus, farmers can get early information about the disease pathogen, which can contribute to good decision-making about a strategy of disease management. To summarize, results from this study demonstrate the usefulness of the PCR-based molecular detection of *Dickeya* sp. (*P. chrysanthemi*) in banana production.

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