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Rapid and Sensitive Detection of the Causal Agents of Postharvest Kiwifruit Rot, *Botryosphaeria dothidea* and *Diaporthe eres*, Using a Recombinase Polymerase Amplification Assay

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The occurrence of postharvest kiwifruit rot has caused great economic losses in major kiwifruit-producing countries. Several pathogens are involved in kiwifruit rot, notably *Botryosphaeria dothidea*, and *Diaporthe* species. In this study, a recombinase polymerase amplification (RPA) assay was developed for the rapid and sensitive detection of the pathogens responsible for posing significant threats to the kiwifruit industries. The RPA primer pairs tested in this study were highly specific for detection of *B. dothidea* and *D. eres*. The detection limits of our RPA assays were approximately two picograms of fungal genomic DNA. The optimal conditions for the RPA assays were determined to be at a temperature of 39°C maintained for a minimum duration of 5 min. We were able to detect the pathogens from kiwifruit samples inoculated with a very small number of conidia. The RPA assays enabled specific, sensitive, and rapid detection of *B. dothidea* and *D. eres*, the primary pathogens responsible for kiwifruit rots in South Korea.

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Kiwifruit (*Actinidia chinensis*) is one of the most popular fruits worldwide owing to its rich source of vitamin C, balanced nutritional components of minerals, and low calories which contribute to their health effects (Li et al., 2017; Stonehouse et al., 2013). However, when exporting kiwifruits, there are problems including postharvest rots, which cause significant losses during storage, distribution, and the shelf-life period (Manning et al., 2016). In general, the commencement of domestic kiwifruit harvesting occurs around mid-October and extends until April, thanks to the utilization of low-temperature storage (Choi et al., 2019). During the storage of kiwifruit, various fungal pathogens deteriorate the quality of fruits and cause postharvest fruit rots (Kim and Koh, 2018; Shin et al., 2021).

Postharvest fruit rots of kiwifruit have been related to more than seven fungi (Beraha and O'Brien, 1979; Hawthorne et al., 1982; Pennycook, 1985). Among them, *Botryosphaeria* spp. and *Diaporthe* spp. were found to be the primary cause of the kiwifruit rots (Du et al., 2021). *Botryosphaeria dothidea* is usually isolated from the postharvest fruit rots of kiwifruit, which was frequently reported in New Zealand, South Korea, and China. In addition, *B. dothidea* is a fungal pathogen capable of invading the kiwifruit via undamaged pericarp through natural openings (Li et al., 2017; Marsberg et al., 2017). Among the major pathogens that cause kiwifruit rots, *Diaporthe* spp. (anamorph: *Phomopsis* spp.) is a significant pathogen. *Diaporthe eres* has been reported in association with postharvest fruit rot in hardy kiwifruit in China, causing severe disease and thus significant economic losses in Northeast

China (Liu et al., 2021). In recent years, *D. eres* is reported to cause the rotting of kiwifruit (*A. deliciosa*) in South Korea (Gi et al., 2022).

Several diagnostic methods have been developed to solve the problem of kiwifruit rots. Polymerase chain reaction (PCR) is required to verify the PCR amplicons increasing the overall cost and time expenditure (Wambua et al., 2017). Recent research has shown the availability of isothermal nucleic acid amplification, such as loop-mediated isothermal amplification (LAMP), which has also been applied for detection of kiwifruit rots pathogens (Qian et al., 2018; Wang et al., 2021). It requires four to six primers to increase specificity as well as isothermal devices, such as heat blocks for incubating at 60-65°C (Lu et al., 2021). Compared with conventional PCR and LAMP methods, the recombinase polymerase amplification (RPA) assay has been proven to be specific, sensitive, rapid, and cost-effective (Lobato and O'Sullivan, 2018). In this study, we developed a rapid and highly sensitive RPA assay for detection of *B. dothidea* and *D. eres*, respectively, to ensure effective control of fruit rots throughout the stages of cultivation, storage, and distribution. We compared the specificity and sensitivity of the RPA assay to those of the conventional PCR. In addition, we evaluated the effectiveness of the RPA assay by detecting *B. dothidea* and *D. eres* in kiwifruits showing rotting symptoms.

In 2019, fungal isolates were obtained from kiwifruits (cv. Hayward) grown in orchards located in Boseong County, South Korea. Kiwifruits with typical postharvest rot symptoms were treated with 70% ethanol for 60 s to disinfect the surface of kiwifruits, then rinsed for 60 s in sterile distilled water, and air-dried for 1 h. Skins were peeled and four pieces of flesh (1 cm^2) were separated from the margin of symptomatic tissues and cultured on potato dextrose agar media (PDA) and grown for 6 days at 25°C. After incubation, a mycelial plug was transferred to fresh PDA and incubated at 25°C for 7 days. To obtain pure cultures of *B. dothidea* and *D. eres*, sporulation was achieved in a modified Barley media (Kim and Park, 1998). After 10 days, pycnidia were produced on the Barley media. Barley seeds with pycnidia were immersed in sterilized water for 10 min and mycelial fragments were removed by filtering through sterile Miracloth (Merck, Rahway, NJ, USA).

Genomic DNA (gDNA) was extracted from PDA culture of *B. dothidea* and *D. eres,* using the i-genomic BYE DNA extraction mini kit (iNtRON, Seongnam, Korea). For molecular identification of fungal isolates, internal transcribed spacer and rDNA region were amplified using the universal primer pair, ITS4 (5′-TCC TCC GCT TAT TGA TTG ATT GC-3′) and ITS5 (5′-GGA AGT AAA AGT CGT AAC AAG G-3′). Conventional PCR was conducted using 1 μl of fungal gDNA, 1 μl of each primer (20 μM of ITS4 and ITS5), and 7 μl of sterile distilled water to 10 μl of 2× TOPsimple DyeMIX (aliquot)-nTaq pre-mixture (Enzynomics, Daejeon, Korea). The reaction conditions were an initial preheated phase at 95°C for 2 min, followed by 32 cycles of DNA denaturation at 95°C for 30 s, a process of primer annealing at 57°C for 60 s, extension at 72°C for 1 min, and the final extension at 72°C for 5 min. PCR products were detected by electrophoresis using a 1.5% agarose gel. Amplified products were purified using a DNA purification kit (Nucleogen, Siheung, Korea) and were sent to Macrogen Inc. (Daejeon, Korea) for sequencing reactions.

RPA primers for detection of *B. dothidea* and *D. eres* were designed according to the TwistDx RPA User's Guide (TwistDX, Ltd., Cambridge, UK). For *B. dothidea*, sequences of *β*-tubulin (*TUB*) genes (KU565871.1, KC864747.2, KJ801793.1, MG564762.1, MG564761.1, MG564760.1, MG564759.1, MG564758.1, and MG564757.1) were downloaded from the NCBI GenBank. For *D. eres*, sequences of histone H3 (*HIS*) gene (KJ420842.1, KJ420871.1, KP714488.1, KP714489.1, MN224562.1, MH121476.1, OL436035.1, OL436036.1, OL436037.1, and OL436038.1) were downloaded from the NCBI GenBank. RPA primers were designed based on highly conserved regions that were identified by alignment of the collected sequences, using BioEdit (v7.2.5) and Mega version 11 programs (Table 1). RPA reactions were performed in a total volume of 50 μl using a TwistAmp basic RPA kit (TwistDX, Ltd.) following the manufacturer's instructions. The reaction mixture consisted of 2.4 μl of each RPA primer (10 μM), 29.5 μl of 1× rehydration buffer, 2 μl of gDNA, 11.2 μl sterile distilled water, and 2.5 μl of magnesium acetate (280 nM). The mixture was incubated at 39°C for 20 min. After the RPA reaction, 10 μl of amplified products were confirmed by loading on a 1.5% agarose gel.

A total of six primer sets were specifically designed for RPA analyses, targeting *TUB* in *B. dothidea* and *HIS* in *D. eres*. For *TUB* in *B. dothidea*, the primer sets included five primers, TUB_F1, TUB_F2, TUB_R1, TUB_R2, and TUB_R3, and for *HIS* in *D. eres*, the primer sets comprised of five primers, HIS_F1, HIS_F2, HIS_F3, HIS_R1, and HIS_R2 (Table 1, Supplementary Fig. 1). To assess the specificity of these primer sets, we used gDNA extracted from the mycelia of *Botryosphaeria dothidea*, *Diaporthe eres*, *Botrytis cinerea*, *Alternaria alternata*, and *Pestalotiopsis* sp. These fungal isolates were obtained from infected kiwifruits in orchards in Boseong County, South Korea. Both the RPA and conventional PCR analyses demonstrated remarkable specificity, successfully amplifying the

Table 1. RPA primer pairs were designed from the pathogen specific sequence of *TUB* gene of *Botryosphaeria dothidea* and *HIS* gene of *Diaporthe eres*

Species (target gene)	Primer sets	Primer name	Sequence (5′-3′)	Length (bp)	Amplicon size (bp)
B. dothidea (*β*-tubulin)	B. d_1	TUB_F1	ATCATTCTCAGCGTGGGAGAACATCAATGACTAA	34	156
		TUB_R1	CTGCGCGTTCAGAAGATTGCCATACTTTAC	30	
	B. d_2	TUB_F2	GTGGGAGAACATCAATGACTAAACTGTAGCAGC	33	144
		TUB_R2	CTGCGCGTTCAGAAGATTGCCATACTTTACG	31	
	B. d_3	TUB_F1	ATCATTCTCAGCGTGGGAGAACATCAATGACTAA	34	156
		TUB_R3	CTGCGCGTTCAGAAGATTGCCATACTTTACGTGT	34	
	B. d_4	TUB_F1	ATCATTCTCAGCGTGGGAGAACATCAATGACTAA	34	156
		TUB_R2	CTGCGCGTTCAGAAGATTGCCATACTTTACG	31	
	B. d_5	TUB_F2	GTGGGAGAACATCAATGACTAAACTGTAGCAGC	33	143
		TUB_R1	CTGCGCGTTCAGAAGATTGCCATACTTTAC	30	
	B. d_6	TUB_F2	GTGGGAGAACATCAATGACTAAACTGTAGCAGC	33	147
		TUB_R3	CTGCGCGTTCAGAAGATTGCCATACTTTACGTGT	34	
D. eres (histone H3)	D_1	HIS_F1	AGTCCGCGCCCTCCACCGGAGGTGTCAAGAAGC	33	210
		HIS_R1	GATCTCACGGACCTATTGGAGGAGGCGATGA	31	
	D_2	HIS_F2	GTCCGCGCCCTCCACCGGAGGTGTCAAGAAGC	32	209
		HIS_R1	GATCTCACGGACCTATTGGAGGAGGCGATGA	31	
	D_3	HIS_F3	TCCGCGCCCTCCACCGGAGGTGTCAAGAAGC	31	208
		HIS_R1	GATCTCACGGACCTATTGGAGGAGGCGATGA	31	
	D_4	HIS_F1	AGTCCGCGCCCTCCACCGGAGGTGTCAAGAAGC	33	209
		HIS_R2	GATCTCACGGACCTATTGGAGGAGGCGATG	30	
	D_5	HIS_F2	GTCCGCGCCCTCCACCGGAGGTGTCAAGAAGC	32	208
		HIS_R2	GATCTCACGGACCTATTGGAGGAGGCGATG	30	
	D_6	HIS_F3	TCCGCGCCCTCCACCGGAGGTGTCAAGAAGC	31	207
		HIS_R2	GATCTCACGGACCTATTGGAGGAGGCGATG	30	

RPA, recombinase polymerase amplification; TUB, β-tubulin; HIS, histone H3; B. d, *Botryosphaeria dothidea*; D, *Diaporthe eres*.

target genes, *TUB* in *B. dothidea* and *HIS* in *D. eres* (Fig. 1, Supplementary Fig. 2). Out of the six primer sets evaluated, primer set B. d_1 for *TUB* in *B. dothidea* and the primer set D_1 for *HIS* in *D. eres* displayed superior amplification of gDNA from *B. dothidea* and *D. eres*, respectively, while exhibiting minimal background amplification for gDNA derived from other fungal pathogens. Based on these findings, these primer sets were chosen for further optimization

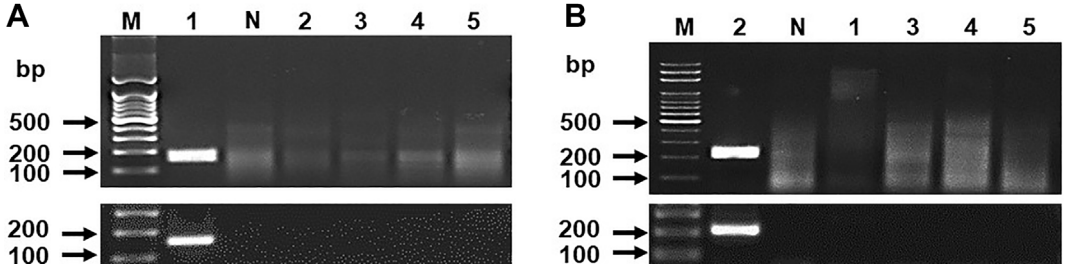

Fig. 1. Specificity of the recombinase polymerase amplification (RPA) assay for *Botryosphaeria dothidea* (A) and *Diaporthe eres* (B). Primer pairs targeting *β*-tubulin and histone H3 were designed for specific detection of *B. dothidea* and *D. eres*, respectively. Gel electrophoresis of RPA products (upper panel) and conventional polymerase chain reaction products (lower panel). Lane 1, *B. dothidea*; lane 2, *D. eres*; lane 3, *Botrytis cinerea*; lane 4, *Alternaria alternata*; lane 5, *Pestalotiopsis* sp.; N, no DNA input; M, DNA ladder.

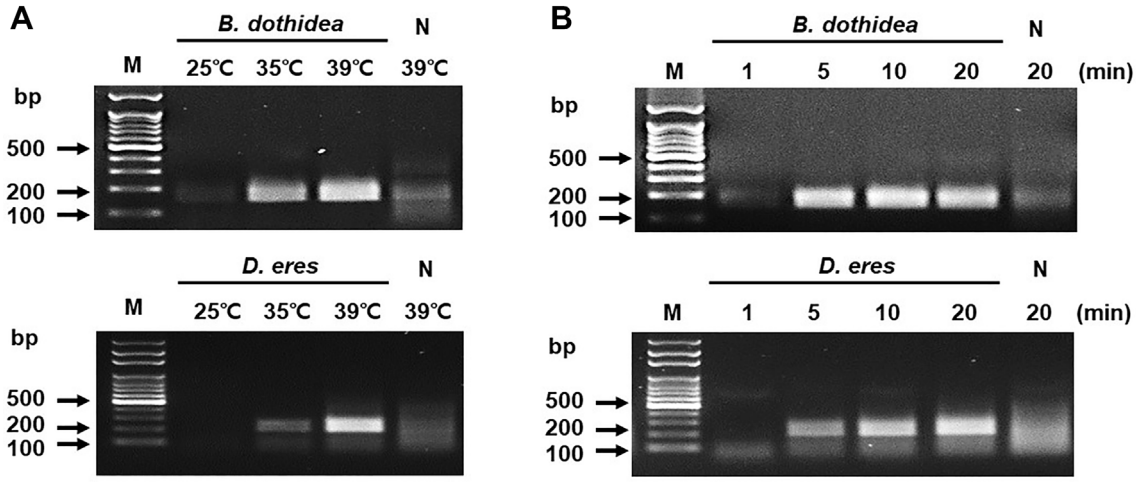

Fig. 2. Optimization of the recombinase polymerase amplification (RPA) assay for *Botryosphaeria dothidea* and *Diaporthe eres*. (A) *β*-tubulin in *B. dothidea* (upper panel) and histone H3 in *D. eres* (lower panel) were amplified from 10 ng of gDNA at different temperature regimes (25°C, 35°C, and 39°C). (B) The RPA assays were performed at a temperature of 39°C and an incubation time of 1, 5, 10, and 20 min for amplification of *β*-tubulin in *B. dothidea* (upper panel) and histone H3 in *D. eres* (lower panel). N, no DNA input; M, DNA ladder.

of the RPA analysis.

Next, the chosen primer sets were evaluated to ascertain the ideal temperature and incubation duration for RPA analysis. Three distinct temperatures, namely 25°C, 35°C, and 39°C, were tested using 10 ng of gDNA, and the RPA reactions were terminated after a 20 min incubation period. Notably, well-defined bands were observed for *TUB* in *B. dothidea* at both 35°C and 39°C, while for *HIS* in *D. eres*, well-formed bands were specifically observed at 39°C (Fig. 2A). In order to identify the ideal incubation time, RPA analyses were performed at a temperature of 39°C. The reactions were halted at different time points: 1 min, 5 min, 10 min, and 20 min after incubation. As a result, distinct and well-defined bands became visible as early as 5 min for both *TUB* in *B. dothidea* and *HIS* in *D. eres*. However, the most favorable incubation time, resulting in the best-defined bands, was observed after 20 min (Fig. 2B). Taken together, the RPA assays were optimized to achieve the best results by maintaining an incubation temperature of 39°C for a minimum of 5 min.

To assess the sensitivity of the RPA assay, a series of 10-fold dilutions was prepared for both *B. dothidea* and *D. eres* gDNA. The dilution series covered a range from 12.6 ng/μl to 1.26 pg/μl for *B. dothidea* gDNA and from 8.4 ng/μl to 0.84 pg/μl for *D. eres* gDNA. Well-defined bands were observed for all the reactions added with 2 μl of the dilution series of gDNA, and there was no amplification without input gDNA (Fig. 3). In comparison to the conventional PCR results, the RPA assay exhibited superior sensitivity. Specifically, for *TUB* in *B. dothidea*, the RPA assay demonstrated a sensitivity that was at least 1,000 times greater (Fig. 3A), while for *HIS* in *D. eres*, it was 100 times more sensitive (Fig. 3B).

In order to evaluate the effectiveness of the RPA assay,

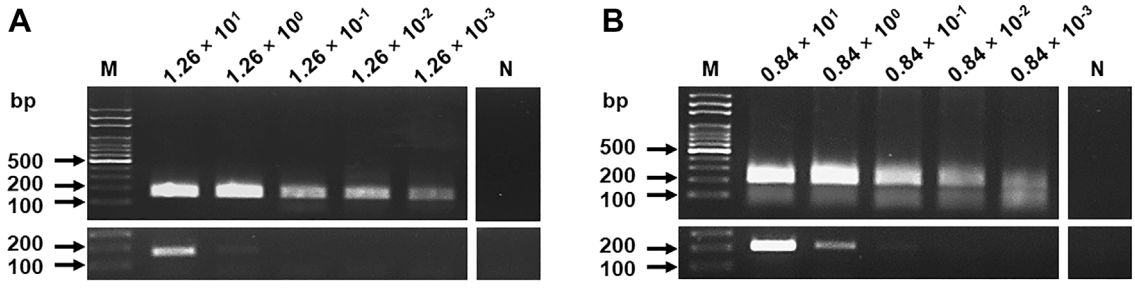

Fig. 3. Sensitivity of the recombinase polymerase amplification (RPA) assay for *Botryosphaeria dothidea* (A) and *Diaporthe eres* (B). Two microliters of a 10-fold dilution series of *B. dothidea* and *D. eres* gDNA (ng/μl) were subjected to the RPA assay at 39°C for 20 min. N, no DNA input; M, DNA ladder.

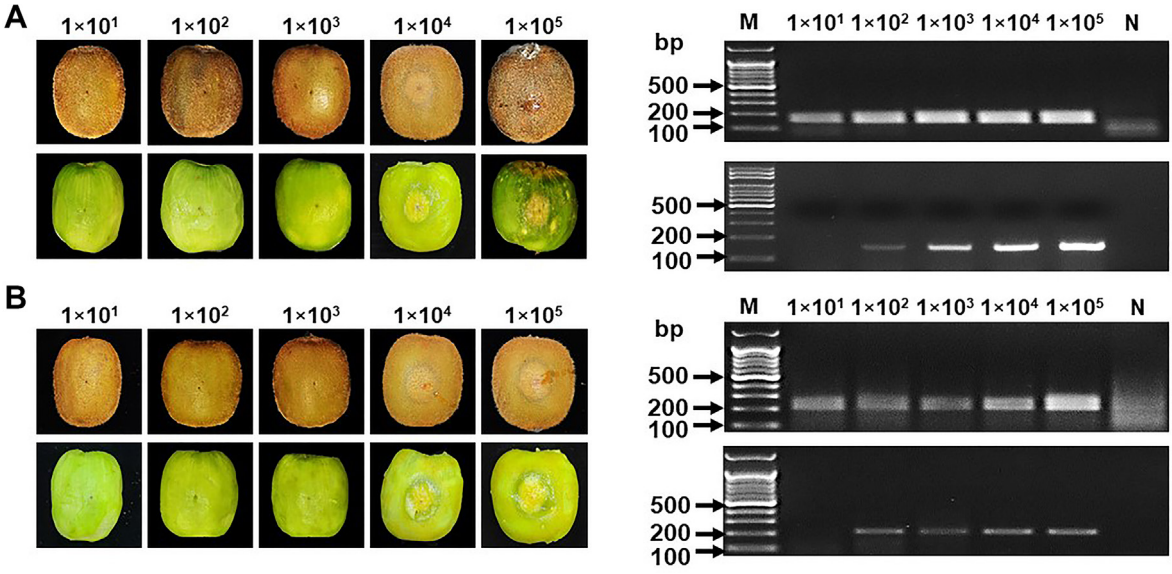

Fig. 4. Efficacy of the recombinase polymerase amplification (RPA) assay for *Botryosphaeria dothidea* (A) and *Diaporthe eres* (B). Kiwifruits were inoculated with the indicated concentrations of conidia suspension (spore numbers/ml) and the photos were taken 10 days after inoculation (left panels). Two microliters of gDNA extracted from kiwifruit skin peeled from the inoculation site were subjected to the RPA assay at 39°C for 20 min (right upper panels) and conventional polymerase chain reaction assay (right lower panels). N, no DNA input; M, DNA ladder.

healthy kiwifruits were inoculated with varying concentrations of conidia of *B. dothidea* and *D. eres* and subjected to both RPA and conventional PCR analyses. Conidia of *B. dothidea* and *D. eres* were filtered and adjusted to concentrations ranging from 1×10^1 conidia/ml to 1×10^5 conidia/ml. Healthy kiwifruits, obtained from a grocery store, were wounded and inoculated with 20 µl of the spore suspensions on the fruit surface. The inoculated fruits were then placed in a chamber and incubated at 25°C. Following a 10 days incubation period, the peel of the kiwifruits at the site of inoculation was carefully removed and subjected to gDNA extraction. Subsequently, a 2 µl volume of extracted gDNA was used for RPA and conventional PCR analyses. Genomic DNA extracted from kiwifruit samples that had been wounded, but not inoculated with spores was used as a negative control (Fig. 4). The RPA assays were successful in detection of *B. dothidea* and *D. eres* in kiwifruit showing typical rot symptoms, as well as in asymptomatic kiwifruits that had been inoculated with a very small number of conidia.

Kiwifruit production suffers greatly from postharvest fruit rot that leads to significant economic losses for kiwifruit farms throughout the harvesting, storage, distribution, and marketing processes (Hawthorne et al., 1982). In South Korea, there has been a lack of research concerning fruit rots in kiwifruit and effective methods for identifying infected fruits. During storage at low temperatures after harvesting, it is crucial to exercise great caution in order to prevent the spread of postharvest fruit rot diseases to healthy kiwifruits. However, it is very challenging to distinguish infected kiwifruit due to long latent periods. Therefore, it becomes necessary to develop molecular diagnostic techniques to detect the pathogens responsible for kiwifruit rots. The causal agents of kiwifruit rots, namely *B. dothidea* and *D. eres* quickly destroy ripening kiwifruits, emphasizing the need for a simple, rapid, sensitive, and accurate method like the developed RPA method to detect these pathogens. In the current study, the RPA assay demonstrated superior speed and specificity in detecting *B. dothidea* and *D. eres* in infected kiwifruits compared to the conventional PCR assay. The assay exhibited rapid performance, taking only 5 min, and could be conducted at a relatively low temperature of 39°C. As a result, it holds great promise as a diagnostic method for identifying infected kiwifruits during storage and transportation, enables effective controls of kiwifruit rot disease, and contributes to extending the shelf-life period of kiwifruits.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

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Electronic Supplementary Material

Supplementary materials are available at The Plant Pathology Journal website (http://www.ppjonline.org/).

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