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Comparison of different methods for total RNA extraction from sclerotia of *Rhizoctonia solani*

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ABSTRACT

Background: Rhizoctonia solani (teleomorph: *Thanatephorus cucumeris*) is one of the most important pathogens of rice (*Oryza sativa* L.) that causes severe yield losses in all rice-growing regions. Sclerotia, formed from the aggregation of hyphae, are important structures in the life cycles of *R. solani* and contain a large quantity of polysaccharides, lipids, proteins and pigments. In order to extract high-quality total RNA from the sclerotia of *R. solani*, five methods, including E.Z.N.A.™ Fungal RNA Kit, sodium dodecyl sulfate (SDS)–sodium borate, SDS–polyvinylpyrrolidone (PVP), guanidinium thiocyanate (GTC) and modified Trizol, were compared in this study.
Results: The electrophoresis results showed that it failed to extract total RNA from the sclerotia using modified Trizol method, whereas it could extract total RNA from the sclerotia using other four methods. Further experiments confirmed that the total RNA extracted using SDS–sodium borate, SDS–PVP and E.Z.N.A.™ Fungal RNA Kit methods could be used for RT-PCR of the specific amplification of GAPDH gene fragments, and that extracted using GTC method did not fulfill the requirement for above-mentioned RT-PCR experiment.
Conclusion: It is concluded that SDS–sodium borate and SDS–PVP methods were the better ones for the extraction of high-quality total RNA that could be used for future gene cloning and expression studies, whereas E.Z.N.A.™ Fungal RNA Kit was not taken into consideration when deal with a large quantity of samples because it is expensive and relatively low yield.

1. Introduction

The soil-borne fungus *Rhizoctonia solani* Kühn (teleomorph: *Thanatephorus cucumeris* (Frank) Donk) has been proved to be a destructive and widespread pathogen that infects many plant species, including cereal crops, vegetable crops, ornamental crops, and fruit trees. Sclerotia of *R. solani* develop initially as the aggregation of small groups of hyphae to form loose primordia which arise from the irregular branching and increased septation of neighboring hyphae. Sclerotia serve as dormant structures, which are hard, asexual and resistant to the unfavorable conditions and chemical and biological degradation. *R. solani* produces sclerotia to survive in soil when host plants are absent and might grow and cause disease in the next growing season [1,2,3,4,5,6]. Although considerable literatures have focused on histochemistry, ultrastructure, primary and secondary metabolism, as well as the effect factors and conditions for the formation and development of sclerotia, very little is known about the molecular mechanisms involved in sclerotial formation and development of *R. solani* [1,3,4,7,8,9,10].

High quality RNA is important for molecular biology research such as cDNA library construction, SSH (suppression subtractive hybridization), RT-PCR, cDNA-AFLP and Northern blot [11,12,13,14]. However, to extract high quality RNA from filamentous fungi is difficult because filamentous fungi are rich in polysaccharides and phenolic compounds. Once the disruption of fungal cells by SDS or GTC, the phenolic compounds oxidize readily, link covalently with quinones and bind to nucleic acids irreversibly [15,16,17]. As a result, the co-precipitation of polysaccharides with RNA and the oxidation of phenolic compounds cause the browning effect and make RNA difficult to be dissolved [18,19,20,21,22,23,24]. As a result, polyphenols are left over in the extraction process which may decrease RNA yield, as well as inhibit PCR amplification.

In order to overcome the oxidation problem, there are many anti-oxidation compounds which can be incorporated to RNA extraction buffers. Among anti-oxidation compounds, it may be named polyvynilpyrrolidone (PVP), sodium borate, 2-mercaptoethanol and others. The lysis buffer contained a high concentration of sodium borate, which forms H-bonded complexes with polyphenols and inhibit the oxidation of polyphenol compounds in the early steps. Then the complexes can be effectively removed by high-speed centrifugation

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[25]. PVP plays a role in removing phenolic compounds and secondary metabolites from nucleic acid preparations [20,22], and it also prevents browning effect of polyphenols. It has been reported that PVP can strongly bind to the polyphenol compounds by its CO – N = group [26] and as the numbers of aromatic groups increase, the binding function of the PVP increases. Then, chloroform:isoamyl alcohol is used to remove PVP before addition of PCI since PVP is not compatible with phenol [23].

As sclerotia of *R. solani* formed from filamentous tissue, the cytoplasms of sclerotia consist of abundant protein, polysaccharide, lipid, glycogen and polyphosphate, and the cell walls contain glucans and chitin [7]. For this reason, it is more difficult to extract high-quality RNA from sclerotia than do it from mycelia of *R. solani*. In the previous study, RNA was successfully extracted from sclerotia of *Sclerotinia sclerotiorum* with Trizol reagent using standard protocols by Rollins [27]. However, relevant methods that can steadily extract high-quality RNA from sclerotia of *R. solani* have not been reported so far.

The main objective of this study was to find out the best and reliable RNA extraction method(s) so as to extract high quality RNA from sclerotia of *R. solani* for the downstream research in fungal genomics and transcriptomics. At present, the availability of the genome sequence of *R. solani* provides an invaluable tool for hypothesis-driven study on sclerotium biology. Identifying the molecular factors affecting sclerotial development and evaluating their functions will provide a better understanding of the basic physiological and morphological processes involved. For the study of gene expression in sclerotia of *R. solani*, the extraction of RNA is crucial, because it is a pre-requisite for diverse molecular techniques such as isolating genes by RT-PCR, RNA-seq, southern blot analysis and the construction of representative cDNA libraries. For this purpose, five RNA extraction methods, *i.e.* E.Z.N.A.™ Fungal RNA Kit, SDS–sodium borate, SDS–PVP, GTC and modified Trizol, were compared in this study.

2. Materials and methods

2.1. Fungal strain and sclerotia collection

The virulent strain GD-118 of *R. solani* AG-1 IA, the causal agent of rice sheath blight, maintained by our laboratory [28,29,30], was used in this study. The strain GD-118 was incubated on potato dextrose agar (PDA) plates at 28°C for 14 days, and the black sclerotia were collected with forceps and stored at -80°C until use.

2.2. Reagents

E.Z.N.A.™ Fungal RNA Kit (Omega, GA, USA); SDS–sodium borate solution: 2% SDS, 0.025 M sodium borate (pH8.5); SDS–PVP solution: 0.25 M NaCl, 0.05 M Tris·HCl (pH 7.5), 20 mM EDTA, 1% SDS, 4% soluble PVP K-30; GTC solution: 6 M GTC, 37.5 mM citric acid, 0.75% N-lauroyl sarcosine sodium, 0.15 M β-mercaptoethanol (which should be added into GTC solution just before use); Trizol reagent (Invitrogen, CA, USA). All the other analytical reagents used in this study were purchased from Sangon Biotech Co. Ltd. (Sangon, Shanghai, China).

2.3. RNA extraction and analysis

2.3.1. E.Z.N.A.™ Fungal RNA kit method

Extraction of total RNA from sclerotia of *R. solani* was performed according to the manufacturer's recommendations.

2.3.2. SDS–sodium borate method

The protocol was based on the method described by López-Gómez and Gómez-Lim [25] with minor modification. 100 mg of frozen sclerotia was ground into fine powder in the presence of liquid nitrogen and silica sand. The resulting powder was extracted twice with 1 ml 70% acetone pre-colded at -80°C with centrifugation at 8000 *g* for 5 min at 4°C. The pellets were mixed with 0.6 ml SDS–sodium borate solution, 60 μl β-mercaptoethanol, 0.4 ml Tris–phenol (pH 8.0) and 0.3 ml chloroform and agitated vigorously for 3 min, followed by centrifugation at 12,000 *g* for 10 min at 4°C. The supernatant was mixed with 0.3 ml Tris–phenol (pH 8.0) and 0.3 ml chloroform with occasional agitation for 2 min, followed by another centrifugation at 12,000 *g* for 10 min at 4°C. The supernatant was extracted with equal volume of chloroform and centrifuged at 12,000 *g* for 10 min at 4°C. To remove the polysaccharides, the aqueous phase was mixed with 1/10 volume of chilled 3 M NaAc (pH 5.2) and 1/10 volume of chilled absolute ethanol, and then the tube was vibrated sufficiently and centrifuged at 12,000 *g* for 5 min at 4°C. The RNA was precipitated with equal volume of 4 M LiCl and a half volume of chilled absolute ethanol, and was collected by centrifugation at 12,000 *g* for 10 min at 4°C. The pellet was dissolved in 400 μl RNase-free water subsequently, and mixed with 1/10 volume of chilled 3 M NaAc (pH 5.2) and 3-fold volume of chilled absolute ethanol. The mixture was incubated at -20°C for at least 30 min, and centrifuged at 12,000 *g* for 15 min at 4°C. The RNA pellet was washed with 75% (v/v) ethanol, dissolved in 30 μl of RNase-free water and stored at -80°C.

2.3.3. SDS–PVP method

The protocol was modified from Chan et al. [22]. 100 mg of frozen sclerotia was treated twice with acetone as described in above-mentioned SDS–sodium borate method. After extraction, the precipitate was mixed with 0.75 ml SDS–PVP solution and 0.75 ml chloroform/isoamyl alcohol (24:1, v/v) and vortexed drastically, followed by centrifugation at 12,000 *g* for 2 min at room temperature. The supernatant was extracted twice with equal volume phenol/chloroform/isoamyl alcohol (PCI) (25:24:1, v/v/v) adequately and centrifuged at 12,000 *g* at room temperature for 5 min. Then, the aqueous phase was mixed with 1/10 volume of chilled 3 M NaAc (pH 5.2) and 1/10 volume of chilled absolute ethanol, and the tube was vibrated sufficiently and centrifuged at 12,000 *g* for 5 min at 4°C. To precipitate RNA, the aqueous phase was mixed with 1/10 volume of chilled 3 M NaAc (pH 5.2) and 3-fold volume of chilled absolute ethanol. The tube was incubated at 4°C for at least 30 min, and centrifuged at 12,000 *g* for 20 min at 4°C. The pellet was washed with 70% (v/v) ethanol once and dissolved in 200 μl of DEPC treated water and mixed with 50 μl of 10 M LiCl adjusted to final concentration of 2 M. The mixture was precipitated at -20°C for at least 1 h and centrifuged at 12,000 *g* for 20 min at 4°C. The RNA pellet was washed with 75% (v/v) ethanol, resuspended in 30 μl of RNase-free water and stored at -80°C.

2.3.4. GTC method

The procedure was based on the method described by Yao et al. [31] with minor modification. 100 mg of frozen sclerotia was treated twice with acetone as described in SDS–sodium borate method. The pellet was mixed with 0.6 ml GTC solution thoughtfully; 150 μl 2 M NaAc (pH 4.0) was added subsequently and reversed mixing gently. Then, 500 μl PCI was added and agitated vigorously for 10 s and cooled on ice for 15 min, followed by centrifugation at 12,000 *g*, 4°C for 5 min. The upper clear aqueous layer was extracted with 500 μl of PCI once more. The supernatant was mixed with equal volume of chilled isoamylol and incubated at -20°C for 1 h. After centrifugation at 12,000 *g*, 4°C for 20 min, the precipitation was washed with 75% (v/v) ethanol, dissolved in 30 μl of RNase-free water and stored at -80 °C.

2.3.5. Modified Trizol method

Total RNA was extracted with Trizol reagent (Invitrogen, CA, USA) according to the manufacturer's instructions with some modifications. 100 mg of frozen sclerotia was ground into fine powder and transferred into a 1.5 ml Eppendorf tube, and then 1 ml of Trizol solution was added

Table 1
Purity and yield analysis of total RNA extracted by different methods.

Method	OD_{260}/OD_{280}	Yield of RNA (μg/g)
E.Z.N.A.™ Fungal RNA Kit	1.977 ± 0.071	72.84 ± 1.75
SDS–sodium borate	2.013 ± 0.102	182.5 ± 2.74
SDS–PVP	1.832 ± 0.032	134.5 ± 1.96
GTC	1.661 ± 0.021	79.71 ± 1.20
Modified Trizol	–	–

Note: "–" indicates the absence of RNA.

and agitated vigorously for 30 s. The tube was placed on the clean benches for 10 min at room temperature followed by centrifugation at 12,000 *g* for 15 min at 4°C. The supernatant was mixed with equal volume of chilled Tris–phenol (pH 8.0) and isopropanol by reversed mixing for 2 min and centrifuged at 12,000 *g* for 5 min at 4°C. The upper clear aqueous layer was extracted with equal volume of chloroform and centrifuged at 12,000 *g*, 4°C for 5 min. The supernatant was mixed with 0.5 volume of chilled isopropanol and incubated at room temperature for 10 min. The precipitated RNA was collected from the supernatant by centrifugation at 12,000 *g*, 4°C for 20 min. The sediment was washed with of 75% (v/v) ethanol and air-dried for about 10 min. The purified RNA was dissolved in 30 μl of RNase-free water and stored at -80°C.

2.4. Quantitative and qualitative analyses of RNA

Quantitative analysis of RNA was conducted by measuring optical density at 260 nm and 280 nm using a Nanodrop2000 Ultraviolet Spectrophotometer (Thermo Fisher, MA, USA). A pure RNA sample was indicated by a ratio of 2:1 at 260 nm and 280 nm. Total RNA solution was loaded on 1% agarose gel, and visualized under UV light in Alpha Imager HP System (ProteinSimple, CA, USA) to assess the integrity of total RNA bands.

2.5. RT-PCR analysis

RNA samples were subjected to DNase I treatment according to TaKaRa protocol (TaKaRa, Kyoto, Japan). Then the first strand cDNA was synthesized using First Strand Synthesis Kit (Invitrogen, CA, USA) followed the manufacturer's instructions. To verify whether the RNA was suitable for RT-PCR, amplification was performed using specific primers designed from the GAPDH gene of *R. solani*. The primer sequences were: GAPDH F: 5′-TACTCCgCAATgCTATCg-3′; GAPDH R: 5′-TACTCggTCCCAgTggT-3′. RT-PCR was conducted using the following program: 95°C for 2 min followed by 35 cycles of (denaturing at 94°C for 40 s, annealing at 54°C for 40 s, and extension at 72°C for 1 min), with a final extension step at 72°C for 10 min. A 'control PCR' was performed with 1 μg of total RNA without reverse transcription so as to check for genomic DNA contamination. The PCR product was electrophoretically separated on 1% agarose gel. The gel was visualized and photographed under UV light in Alpha Imager HP System (ProteinSimple, CA, USA). The intact band was recovered from agarose gel and cloned into the vector PMD20-T (TaKaRa, Kyoto, Japan) as described by the manufacturer. The recombinant plasmid was transformed into *Escherichia coli* DH5α competent cell. Five single clones carrying recombinant plasmids were randomly selected and sent to AuGCT DNA-SYN Biotechnology Company (AuGCT, Beijing, China) for sequencing.

3. Results and discussion

The success of total RNA extraction should be verified by testing the quantity, quality and integrity of RNA. Table 1 showed the yields of total RNA (μg/g dry wt.) extracted from sclerotia of *R. solani*. All extraction methods except the modified Trizol method described herein were efficient in producing high-quality RNA with a high output from sclerotia of *R. solani* (Fig. 1, Table 1). The yield of RNA extracted by SDS–sodium borate method was the highest at the rate of 182.5 ± 2.74 μg/g.

The quality of extracted total RNA described in this study was judged in three ways. Firstly, the ratios of A260/280 for the E.Z.N.A.™ Fungal RNA Kit, SDS–sodium borate and SDS–PVP methods ranged from 1.86 to 2.01, while the ratio for GTC method was only 1.66, which may be due to a large amount of visible flocculent co-precipitation when isopropanol was added. Secondly, 1.0% agarose gel electrophoresis showed that 28S and 18S bands were intact and bright, demonstrating that the total RNA, extracted by E.Z.N.A.™ Fungal RNA Kit, SDS–sodium borate and SDS–PVP methods, was not degradation (Fig. 1). However, the 28S bands with apparent degradation were observed in GTC method. Thirdly, the quality of total RNA was proved by RT-PCR. As indicated in Fig. 2, when the total RNA extracted by E.Z.N.A.™ Fungal RNA Kit, SDS–sodium borate and SDS–PVP methods described in this study served as a robust template for RT-PCR, the expected amplification of a 256-bp amplicon was obtained. However, no amplification was gained when the total RNA extracted by GTC method was used as template. This might be due to a lower A260/A280 ratio of the total RNA, which was lower than 1.8, indicating that the total RNA extracted by GTC method was impure which could inhibit the activity of the *Taq* DNA polymerase since the reverse transcription was highly sensitive to the impurity of RNA template [23]. The resulting RNA was not contaminated by gDNA as confirmed by the absence of PCR amplification product using the robust RNA samples with the same primers.

Numerous papers have described methods for extracting RNA from diverse organisms including filamentous fungi, but only a few papers dealt with sclerotia of fungi. Bahloul and Burkard [32] extracted RNA from needles, shoots, and callus of spruce, which used soluble polyvinylpyrrolidone (PVP) and lithium chloride precipitation. Schultz et al. [19] developed a new RNA extraction method from recalcitrant tissues such as geranium tissues, mature needles of white pine (*Pinus strobus*) and mature leaves of poinsettia (*Euphorbia pulcherrima*). In

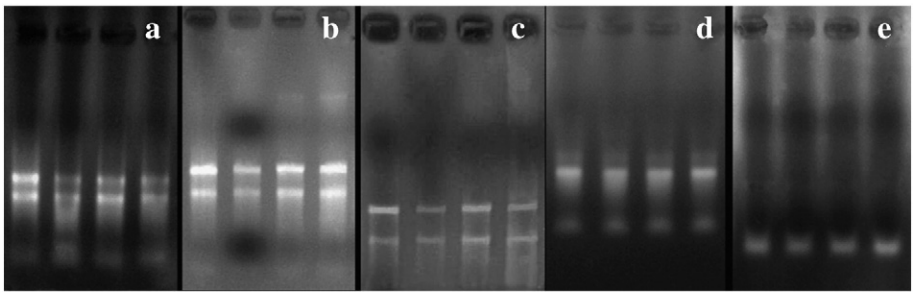

Fig. 1. Electrophorogram of total RNA samples extracted by different methods. (a) E.Z.N.A.™ Fungal RNA Kit; (b) SDS–sodium borate method; (c) SDS–PVP method; (d) GTC method; and (e) modified Trizol method.

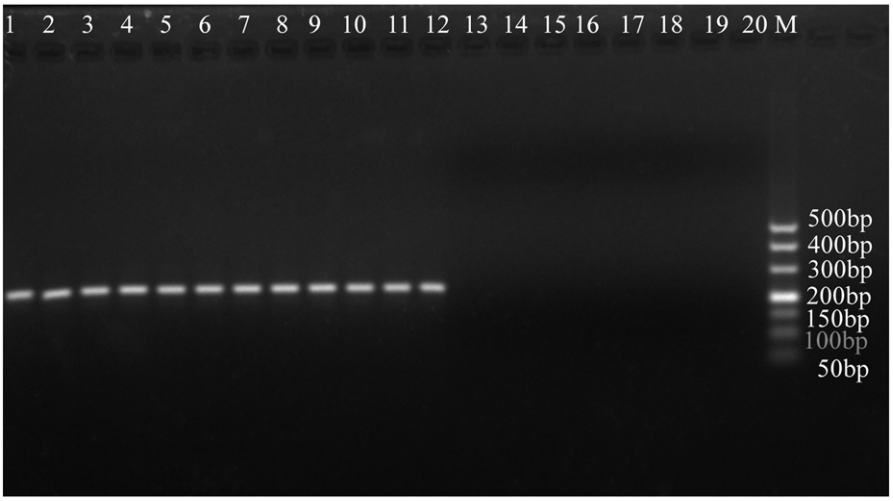

Fig. 2. Electrophorogram of the RT–PCR products. 1–4. E.Z.N.A. ™ Fungal RNA Kit; 5–8. SDS–sodium borate method; 9–12. SDS–PVP method; 13–16. GTC method; 17–20, Modified Trizol method; M. DNA Marker (DL2000).

order to extract RNA form *Gracilaria tenuistipitata*, Falcão et al. [33] found that the best method was the previous treatment with 6.5 M guanidinium hydrochloride extraction buffer and 0.2 M KOAc to remove the polysaccharides. Other RNA extraction protocols for organisms include: the use of hot/SDS and hot/phenol [34]; the use of higher-strength SDS in lysis buffer [14,23]. As mentioned above, sclerotia are hard structures and rich in polysaccharides, phenolic compounds, protein, lipids, secondary metabolites and pigments, which severely interfere with RNA extraction. Although Rollins [27] has successfully extracted RNA from sclerotia of *S. sclerotiorum* using conventional Trizol method, we failed to extract RNA from sclerotia of *R. solani* by using the same method. Interestingly, RNA from mycelia of *R. solani* was relatively easy to be extracted by conventional Trizol methods (data not shown), indicating that RNA extraction methods should be adjusted greatly when dealing with different fungal genera or different structures in the same fungal genus [35].

It is very critical to choose the right detergent in order to obtain high-quality RNA. In this study, guanidinium isothiocyanate was used as detergent in GTC and modified Trizol methods, whereas SDS was used in SDS–sodium borate and SDS–PVP methods. It is reported that chaotropic salts of guanidinium could inhibit RNase activity, but could not be suitable for the tissues rich in polysaccharides or phenolics. Li et al. [36] extracted RNA from *Lentinola edodes* by using guanidinium based methods, which resulted in a large quantity of polysaccharides residue and only very low yields of impure RNA. In the present study, use of high molar concentration of GTC produced highly viscous substance and hence RNA recovery was very difficult. The A260/280 ratio was low (Table 1), and the total RNA obtained from this protocol was not intact (Fig. 1).

SDS lyses the fungal cell wall and inhibits the endogenous and exogenous RNase activity, so it could decrease RNA degradation at relatively higher temperature. In the present study, we obtained intact and high-quality RNA from sclerotia of *R. solani* when using two SDS/phenol methods with modifications. Our results were accord with those reported for *L. edodes* and *Trichoderma* sp. using SDS/phenol method [36,37].

For effective extraction of intact RNA from tissues rich in polysaccharides, it is important to prevent these contaminating substances from binding to nucleic acids. Liu et al. [38] used ice cold potassium acetate to precipitate genomic DNA and the secondary metabolites from the banana fruit tissue. However, Asif et al. [39] found that this step greatly affected RNA yield and developed an optimized polysaccharide precipitation step using 0.1 volume of ethanol and 1/30 volume of 3 M NaAc (pH 5.2). In our study, 1/10 volume of 3 M NaAc (pH 5.2) and 1/10 volume of ethanol were applied to remove the polysaccharides selectively by forming a jelly-like precipitate without affecting the yield of RNA. RNA remained in the supernatant can be precipitated by the addition of 3 M NaAc (pH 5.2) to a final concentration of 0.3 M and 2.5 volumes of absolute ethanol or 10 M LiCl to a final concentration of 2 M. These steps consistently obtained high-quality RNA with the yield ranged from 134.5 to 182.5 μg/g.

The success of total RNA extraction from sclerotia to a great extent depended on the thorough grinding of the fungal tissues. Liquid nitrogen was added duly to prevent the powder to dissolve, whereas the addition of liquid nitrogen during grinding could cause splashing and result in sample loss. One of another minor modifications in the present study was that silica sand was added to avoid splashing and supplement the grinding because sclerotia are hard, which could avoid the fine powder adhere to the mortar. The homogenized solution of sclerotia from *R. solani* was relatively viscous and rich in pigment. One modifications presented in SDS–sodium borate, SDS–PVP and GTC methods included a pre-colded (-80°C) acetone extraction [40], which successfully removed most of pigment and improved the quality of RNA.

To summarize, here we have compared five methods for the extraction of total RNA from sclerotia of *R. solani*. Finally, we found two best manual methods, *i.e.* SDS–sodium borate and SDS–PVP methods, for dealing with a large quantity of sclerotial samples, which could save considerable expense when compare with E.Z.N.A.™ Fungal RNA Kit. Total RNA extracted by these two methods was suitable for downstream applications such as reverse transcription, gene amplification and transcriptome research. The yield of total RNA extracted by E.Z.N.A.™ Fungal RNA Kit was lower than those extracted by SDS–sodium borate and SDS–PVP methods, and the commercial kit is too expensive to deal with a large quantity of samples. SDS–sodium borate and SDS–PVP methods are expected to successfully apply when extracting RNA from various fungi materials which have similar sclerotia structures.

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Conflict of Interest

The authors declare that there is no conflict of interest.

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