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Characterization of *Peanut stunt virus* Isolated from Black Locust Tree (*Robinia pseudo-acacia* L.)

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An isolate of *Peanut stunt virus* (PSV) isolated from black locust tree (*Robinia pseudo-acacia* L.) showing severe mosaic and malformation symptoms, was designated as PSV-Rp. PSV-Rp was characterized by the tests of host range, physical properties, RNA and coat protein composition and RT-PCR analysis. Nucleotide sequences of the cucumoviruses CP genes were also used for identification and differentiation of PSV-Rp. Six plant species were used in the host range test of PSV-Rp. PSV-Rp could be differentiated from each *Cucumovirus* strain used as a control by symptoms of the plants. The physical properties of PSV-Rp virus were TIP 65°C, DEP 10^{-3}, and LIP 2~3 days. In dsRNA analysis, PSV-Rp consisted of four dsRNAs, but satellite RNA was not detected. Analysis of the coat proteins by SDS-PAGE showed one major protein band of about 31 kDa. RT-PCR using a part of *Cucumovirus* RNA3 specific primer amplified ~950bp DNA fragments from the crude sap of virus-infected black locust leaves. RFLP analysis of the RT-PCR product could differentiat PSV-Rp from CMV. The nucleotide sequence identity between the PSV-Rp CP and the TAV-P CP genes and the PS-V-RP CP and CMV-Y CP genes were 61.6% and 40.5%, respectively. On the other hand, the nucleotide sequence identity of the PSV-Rp CP gene was 70.9% ~73.4% in comparison with those of PSV subgroup I (PSV-ER and PSV-J) and 67.3% with that of PSV subgroup II (PSV-W). Especially, the nucleotide sequence identity of PSV-Rp CP gene and that of PSV-Mi that was proposed recently as the type member of a novel PSV subgroup III was 92.4%.

Keywords : CP gene, *Peanut stunt virus*, PSV-Rp, PSV subgroup III

Peanut stunt virus (PSV) is a member of the genus *Cucumovirus* in the family *Bromoviridae* (Fauquet et al., 2001). Other members of the genus are *Cucumber mosaic virus* (CMV) and *Tomato aspermy virus* (TAV). PSV, like other cucumoviruses, has tripartite genomes of positive sense single-stranded RNAs. RNAs 1 and 2 of PSV encode the 1a and 2a proteins, respectively, which are required for replication. RNA3 is also dicistronic and encodes the movement protein (MP) and coat protein (CP). The CP gene is expressed from subgenomic RNA (RNA4) and packaged into a single virus particle together with RNA3 (Mushegian and Koonin, 1993). Occasionally, PSV and CMV also package a fifth RNA, designated satellite RNA (satRNA), along with their genomic and subgenomic RNAs (Roossinck et al., 1992). PSV is an economically important pathogen and occurs worldwide in legumes (Mink, 1972; Xu et al., 1986). PSV-E was first described in the United Stated in 1966 (Miller and Troutman, 1966), and then PSV-W was reported in 1969 (Mink et al., 1969). PSV strains and isolates from other regions of the world have been reported and differentiated based on host symptomology and serology (Xu et al., 1986). The complete nucleotide sequence of the RNAs of PSV-J was first reported by Karasawa et al. (1991, 1992). PSV strains classified into two distinct subgroups, subgroup I containing PSV-ER and subgroup II containing PSV-W, based on the RNA sequence RNA3 (Hu et al., 1997). Recently, PSV-Mi was reported to the type member of a novel PSV subgroup III suggested by Yan et al. (2005).

A PSV isolate (PSV-Rp) was isolated from naturally infected black locust trees showing severe mosaic and malformation on the leaves at Chuncheon in Korea. In this paper, we describe some characteristics and identification of PSV-Rp.

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Materials and Methods

Virus sources and propagation. Virus was isolated from a black locust tree (*Robinia pseudo-acacia* L.) showing mosaic and malformation in Chunchon city (Fig. 1). The infected leaves were ground in 0.01 M phosphate buffer (pH 7.0), and the extract was rubbed onto the leaves of young *Chenopodium amaranticolor*. Single local lesions were isolated from the leaves and then propagated to *Nicotiana benthamiana*. The infected plants were then

Fig. 1. Black locust tree showing mosaic and malformation infected with *Peanut stunt virus* (PSV)-Rp.

maintained in a greenhouse (25±2°C). TAV-Kor was Korean isolate isolated from *Chrysanthemum* sp. CMV-Y and CMV-Ls were kindly supplied from Dr. Takanami Y. (Kyushu University, Japan) and Dr. Palukaitis P. (Scottish Crop Research Institute, UK), respectively. PSV-ER was kindly supplied from Dr. Ghabrial A. (University of Kentucky, USA).

Host range test. Host range of Rp-PSV was determined by sap inoculation of systemically infected *N. benthamiana* leaves. The extract of *N. benthamiana* in 0.01 M phosphate buffer was mechanically inoculated on Carborundum-dusted leaves of 6 plant species (Table 1). Inoculation to each test plant was repeated two or three times using at least three plants. After inoculation, plants were kept in the greenhouse at 25±2°C.

Physical properties. Physical properties in vitro of the virus were examined. The properties were assayed on the local lesion hosts according to the methods of Noordam et al. (1973). Determinations of thermal inactivation point (TIP) [untreated (room temperature), 40~90°C], dilution end point (DEP) (undiluted, 10^{-1}~10^{-7}, w/v) and longevity in vitro (LIV) (0~7 days) of virus were tested on *C. amaranticolor.* Each experiment was performed twice.

Virus purification and electron microscopy. PSV-Rp was purified 7~10 days after inoculation from systemically infected leaves of *N. benthamiana* as described by Takanami (1981) with some modifications. The purified particles were examined by TEM. They were stained negatively with 2% phosphotungstic acid (pH 7.0) and visualized.

Analysis of dsRNA and CP. Viral dsRNAs extraction from the black locust tree infected with PSV-Rp was conducted as described by Morris and Dodds (1979) using CF-11 cellulose (Whatman) column chromatography. DsRNAs were analysed by electrophoresis through 6% polyacrylamide gel containing 1xTAE. The dsRNA bands were visualized by Silver Stain Plus Kit (BIO-RAD). For CP analysis, 5 μg of purified virus was placed in an equal volume of 2x Lammli buffer and heated for 10 min at 100°C. The denatured virus proteins were then electrophoresis through 12.5% polyacrylamide gels containing 0.1%SDS (Lammli, 1970). Protein bands were stained with Silver Stain Plus Kit (BIO-RAD).

RT-PCR and RFLP analysis. The viral RNA was extracted from purified virus particles by SDS-phenol-chloroform treatment and ethanol precipitation. Reverse transcription coupled with the polymerase chain reaction (RT-PCR) and restriction fragment length polymorphism (RFLP) for the 3' half region of RNA 3 covering full-length CP gene of cucumoviruses were carried out according to the method of Choi et al. (1999). RT-PCR was in volume 100 μl containing 1 μl of viral RNA, 2 units of *Taq* DNA polymerase (Promega), 10 μl of thermophilic DNA polymerase 10 x Buffer (Promega), 3 μl of 10 mM dNTPs, 20 unit of AMV reverse transcriptase (Promega), 10 μl of 2.5 mM $MgCl_2$ and 60 pmol each of two primers (CPTALL-3 and CPTALL-5). Amplification was performed on a Minicycler (MJ Research) programmed for 54 min at 42°C,

Table 1. Symptoms on indicator plants of *Peanut stunt virus* (PSV-Rp) isolated from *Robinia pseudo-acacia*

Indicator plants	Symptom[a]			
	PSV-Rp	PSV-ER	CMV-Y	TAV-Kor
Chenopodium amaranticolor	L/–	L/–	L/–	L/–
C. quinoa	L/–	L/–	L/–	L/–
Vigna unguiculata	–/M	–/M	L/–	L/–
Nicotiana benthamiana	–/M	–/M	–/M	–/M
N. glutinosa	–/M	–/M	–/YM	–/MT, MF, D
N. clevelandii	–/M	–/M	–/YM	–/MT, MF, D

[a]M, mosaic; L, local lesion; YM, yellow mosaic; MT, mottling; MF, malformation; D, dwarfing; Inoculated leaf/upper leaf.

94°C for 2.5 min and then 35 cycles of 1 min at 94°C, 2 min at 60°C and 2 min at 72°C, followed by 1cycle of 10 min at 72°C. Amplified PCR products were analysed in agarose gel electrophoresis.

For RFLP analysis, amplified RT-PCR products were treated with *Xho*I and *Eco*RV, and subjected to an agarose gel electrophoresis.

Cloning and sequencing of CP cDNA. The amplified RT-PCR products were purified by the Wizard PCR Preps DNA Purification System (Promega). The purified PCR products were ligated into pGEM-T Easy vector (Promega), and the vector was transformed into competent cells of *Escherichia coli* JM109 (Promega). Each transformation was selected by blue-white screening procedure (Sambrook et al., 1989). Putative transformants were screened by digesting recombinant plasmids with *Eco*RI and *Mbo*I. The plasmid that contained cDNA inserts of the correct size was selected and purified for nucleotide sequencing. Nucleotide and the deduced amino acid sequences of RNA3 CP were analyzed and compared with published data using programs of the DNASTAR software package (USA). The GeneBank accession number used in this analysis were: CMV-Y (M57602), CMV-Q (M21464), CMV-As (X77855), CMV-Ls (AF127976), TAV-P (L15335), PSV-J (D00668), PSV-ER (U15730), PSV-W (U31300), and PSV-Mi (AY775057).

Table 2. Crude sap properties of PSV-Rp in infected *Chenopodium amaranticolor*

Property	PSV-Rp	PSV-ER
Thermal inactivation point (°C)	65	70
Dilution end point (w/v)	10^{-3}	10^{-3}-10^{-4}
Longevity in vitro (days)	2-3	3

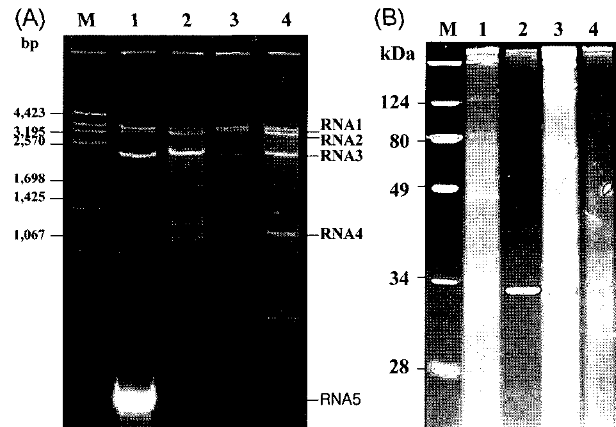

Fig. 2. Electrophoretic profiles of the dsRNA and coat protein (CP). A: 6% PAGE of dsRNAs extracted from the black locust tree leaves infected with PSV-Rp. Lane 1, CMV-Y; Lane 2, TAV-Kor; Lane 3, PSV-ER, Lane 4; PSV-Rp, M, genomic dsRNA of *Rice dwarf virus* (RDV) as a size marker. B: SDS-PAGE analysis of viral coat protein extracted from PSV-Rp (arrow head). Lane 1, CMV-Y; lane 2, TAV-Kor; lane 3, PSV-ER; lane 4, PSV-Rp. M, marker proteins (SDS-PAGE standards, BIO-RAD).

Results and Discussion

Host reaction. The host range and symptoms of PSV-Rp are given in Table 1. Of the indicator plants tested, PSV-Rp systemically infected *N. benthamiana* and some tested plants like as the other cucumoviruses. CMV-Y and TAV-Kor used as control virus developed local lesions on *Vigna unguiculata*, while PSV-ER used as control virus and PSV-Rp systemically infected with the symptom of mosaic.

Physical properties. Crude sap properties of the PSV-Rp are given in Table 2. Thermal inactivation of the PSV-Rp occurred at 65°C which was lower than that of PSV-ER. In 10^{-3} dilution steps, virus infectivity was lost. The longevity in vitro at room temperature was 2~3 days.

DsRNA and coat protein analysis. The sizes and numbers of the dsRNAs extracted from the black locust trees naturally infected with PSV-Rp compared to those of CMV (Y, Ls), TAV-Kor and PSV-ER are shown in Fig. 2A. Molecular sizes of dsRNAs of PSV-Rp were similar to those of CMVs or TAV, but satellite RNA like as CMV-Y was not detected.

When purified preparations of PSV-Rp was examined in the electron microscope, spherical approximately 28 nm diameter particles typical of the cucumovirus group were observed (data not shown). The coat protein of PSV-Rp extracted from purified virus revealed on SDS-PAGE. There was a single protein band about 31 kDa estimated by comigrated molecular marker proteins, and it was corresponded to those of CMV-Y, TAV-Kor and PSV-ER (Fig. 2B).

RFLP analysis of RT-PCR products. To amplify the 3' half region of cucumovirus RNA 3 covering full-length CP gene, RT-PCR was carried out according to the method of Choi et al. (1999). The RT-PCR with the set of primers, CPTALL-3 and CPTALL-5, specifically amplified the target size about 950bp of DNA fragments in PSV-Rp as like as all the tested cucumoviruses (Fig. 3A). RFLP analysis of the PCR products digested with *Xho*I and *Eco*RV showed that PSV-Rp was corresponded to PSV or TAV, and distinguished from CMV-Y and CMV-Ls (Fig. 3, panels B and C).

Sequence comparison and phylogenetic analysis of CP

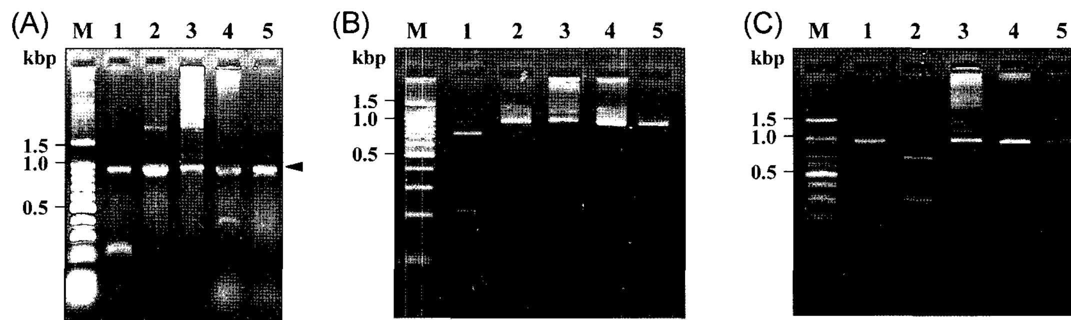

Fig. 3. Agarose gel electrophoresis of the RT-PCR products by *Cucumovirus*-specific primers, CPTALL-5 and CPTALL-3, to amplify the 3' half region of RNA3 covering full-length CP gene from cucumoviruses (A). Arrow head indicates the products amplified by RT-PCR. The RT-PCR products were digested with *Xho*I (B) and *Eco*RV (C), respectively. Lane 1, CMV-Y; Lane 2, CMV-Ls; Lane 3, TAV-Kor; Lane 4, PSV-ER; Lane 5, PSV-Rp; M, 100bp DNA ladder (Bio-Lab).

(A)
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5'-CAGCTTTTAGGTTCAATTCCCTTTGGTCAATTTTGCGTTCCTTTAGCTCCAGGATACTTACCCTGAAACCCTTGTAGTACT
   ATCGTAACCGTTTGTATCGTGTTTACTTTCTACGTGTCAACCACAAGTCGTTATGGCATCTTCAGGATCTGGTAGCGGTT
   CTCGTCGACCCCGCAGGGGAAGACGCAACACACTTACTAAGGTGGATGCTCATGCTAGAGAGCTCCGCGCCCTGACT
   GCTCAGGTGAACCGTTTGGTGGCTATTACTGCTGCCCAGGTGCCATCCCTCGATCATCCAACCTTTGTTTCTAGCAAGA
   AGTGTCGTCCTGGGTACACGTACACTTCGTTGGATGTTAAGCCGGCAAAAACTGAGAAGGGTCAAAGTTTCGGCCAGA
   GGTTATCTTTACCAGCTCCCGTATCCGAATTTCCGAAGAAGAAGATCTCTTGTATTCAGCTCAGGCTGAATCCGTCGCC
   TAAGTTCGACTCCACCATCTGGGTGCCTCTTAGGCGTCTGTCTAAGGACTATTCCCTCTCTTCGGAAAACGTGTTCAAG
   CTCTTCACAGATGGTAACGCAGCAGTGCTTATCTATCAACACGTCTCGACCGGTATTCAACCCCGCAACAAAATTACCT
   TCGATTTAGCCTCCGTTGGAGCGGAAATAGGTGATCTCGGTGAATATGCCGTAATCGTCTATTCCAAGGATGACGTTCT
   GGAGGCCGACGAGATGGTGATACATGTAGATGTGGAGCACCAGCGAATCCCTTCCGCTACAGCTCTCCCGGTCTAGA
   GTTCGGGTATAAGTCCGAAGACTATAAACTACACTCTCTCTGCGAGTGCTAAGTTGGCAGTTATATTACTCCAAACTGTC
   TGAAGTCGCTAAACAGGTTTTCTGGCGAACGGGTTGTCCATCCAGCTGACGGCTAAAATGGTCAGTC-3'
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(B)
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PSV-Mi    1: MASSGSGSGRRPRRGRRNSSAKVDAHARELRALTAQVNRLVAIT
PSV-Rp    1: ···················TLT·······················

PSV-Mi   46: AAQVPSLDHPTFVSSKKCRPGYTYTSLDVKPAKTEKGQSFGKRLS
PSV-Rp   46: ·········································Q···

PSV-Mi   91: LPAPVSEFPKKKISCIQLRLNPSPKFDSTIWVSLRRLPKDYSLAS
PSV-Rp   91: ································P····S·····S·

PSV-Mi  139: ESVFKLFTDGNAAVLIYQHVSTGIQPRNKITFDLASVGAEIGDLG
PSV-Rp  139: ·N···········································

PSV-Mi  187: EYAVIVYSKDDVLEANEMVIHVDVEHQRIPSATALPV
PSV-Rp  187: ···············D·····················
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Fig. 4. Nucleotide sequences of the 3' half region of PSV-Rp RNA3 containing the CP gene (A). The conserved internal control region (ICR) 2-like motif in the intergenic region (IR) is underlined and CP gene is boxed. The 40-nucleotides domain conserved in the 3' untranslated region (UTR) of all cucumovirus RNA sequences is double-underlined. B: Comparison of deduced amino acid sequences from the CP gene of PSV-Rp with those of PSV-Mi. Dots represent amino acid residues that match with PSV-Mi.

gene. The amplified DNA fragment by RT-PCR was cloned and the nucleotide sequence determined. The 3' half region of PSV-Rp RNA3 was 945 nt containing CP gene open reading frame (ORF) consisted of 654 nt (Fig. 4A). The nucleotide sequence identity of CP gene between PSV-Rp and TAV-P, or between that of PSV-Rp and CMV-Y were 61.6% and 40.5%, respectively (Table 3). On the other hand, comparison with the CP gene nucleotide sequences of PSV-J, PSV-ER, PSV-W and PSV-Mi showed they share 73.4%, 70.9%, 67.3% and 92.4% identities with that of PSV-Rp, respectively. In addition, 9 amino acids of PSV-Rp

Table 3. Percent nucleotide sequence identities of the CP gene between PSV-Rp and cucumoviruses

Virus	Identity (%)
PSV-Rp	100%
PSV-J (Subgroup I)	73.4%
PSV-ER (Subgroup I)	70.9%
PSV-W (Subgroup II)	67.3%
PSV-Mi (Subgroup III)	92.4%
TAV-P	61.6%
CMV-Y	40.5%

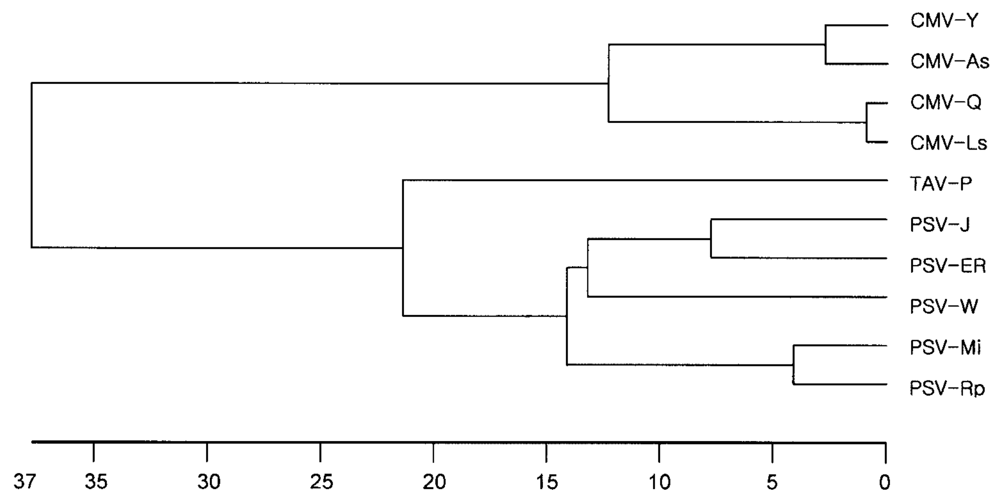

Fig. 5. Phylogenetic relationship of cucumoviruses based on the nucleotide sequence of CP gene.

predicted by the CP gene differed from those of PSV-Mi (Fig. 4B). CMV-As, CMV-Ls, and CMV-Q were included in a phylogenetic analysis of nucleotide sequences identity of the CP genes shown in Fig. 5. In contrast to the CP genes which form three distinct cucumoviruses clusters, PSV-Rp was more closely related to PSV than to TAV or CMV. Also, PSV-Rp is more identical to PSV-Mi which belonged to a novel PSV subgroup III, than to the other PSV subgroups (subgroup I containing J-PSV and ER-PSV and subgroup II containing W-PSV).

This is the first report on the occurrence of PSV from black locust trees (*R. pseudo-acacia*) in Korea. PSV-Rp was characterized based on the host range, biochemical properties, RFLP analysis, and the nucleotide sequence of the CP gene, etc. PSV-Rp induced systemic mosaic in *V. unguiculata*, but CMV-Y and TAV-Kor induced local lesion on inoculated leaves. The 3' half region of RNA 3 covering full-length CP gene of PSV-Rp was successfully amplified by RT-PCR using cucumoviruses specific primers. RFLP analysis of the RT-PCR product with *Xho*I and *Eco*RV could be differentiated PSV-Rp from CMV-Y (subgroup I) and CMV-Ls (subgroup II). The 3' half region of PSV-Rp RNA3 amplified by RT-PCR was 945 nt containing CP gene ORF consisted of 654 nucleotides. The sequence identity of CP gene of PSV-Rp was 73.4% and 70.9% in comparison with that of PSV-J and PSV-ER in PSV subgroup I, 67.3% with that of PSV-W in subgroup II, and 92.4% with that of PSV-Mi in a novel subgroup III. Phylogenetic relationship of the CP genes revealed that PSV-Rp was also closely related to PSV-Mi.

PSV-Mi was identified and characterized in China in 1985, and differentiated from PSV subgroup I and subgroup II by symptomology and serology (Xu and Zhang, 1985). Furthermore, nucleotide sequence analysis of genomic RNAs indicated that PSV-Mi differed sufficiently from other PSV strains of subgroup I and subgroup II to warrant establishment of a novel PSV subgroup III, and PSV-Mi was designated as the type member of the novel PSV subgroup III (Yan et al., 2005).

PSV-Rp and PSV-Mi showed high sequence identity at both the nucleotide and the amino acid levels of CP gene, but showed different reactions on indicator plants. PSV-Mi infected *C. amaranticolor* and *C. quinoa* systemically (Xu et al., 1998), while PSV-Rp (this study) infected these species only locally. The partial nucleotide sequences of RNA3 containing CP gene of PSV-P was also closely related to PSV-Mi, but showed some different reactions on indicator plants. This suggests that neither the coat protein region of the genome nor the coat protein itself is responsible for the differences in symptom severity observed between these two strains (Xu et al., 1998).

Further full-length genomic RNA sequences will be required in order to define characteristics of the PSV-Rp.

Acknowledgments

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