Deciphering the Genetic Identity and Fidelity of Banana Genotype Musa Acuminata through Molecular Fingerprinting

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ABSTRACT

Micropropagation for extensive production of elite planting material clones is an effective and superior alternative to suckers and conventional means of propagation of Musa Spp. The present investigation was consummate to appraise the genetic identity of different micropropagated banana genotypes Grand Naine, Shrimantee and Basrai. Further micropropagated banana genotypes Grand Naine comparing with mother plant during different stages of subculture using two molecular markers SSR (Simple Sequence Repeat) and ISSR (Inter Simple Sequence Repeat). Moreover, for micropropagation explants (suckers) were inoculated on initiation medium containing MS (Murashige and Skoog) fortified with 5 mg/l BAP (Benzyl amino purine) and 39 mg/l AdSO4 (Adenine sulphate). The second, third and fourth subcultures were carried out to separate all multiple shoots. Furthermore, for rooting MS media fortified with growth hormones viz., 2 mg/l BAP, 1mg/l IBA (Indole-3-butyric Acid) and 200mg/l activated charcoal were used under present investigation. The 90 % and 75 % plantlets were successfully established during primary hardening in controlled as well as in natural field condition. Further, 26 primers were screened from that 20 primers (ISSR and SSR) showed 146 clear, reproducible and scorable bands ranging from 100-2000 bp (Base Pair) produced. The banding pattern of these randomly selected plants was monomorphic with mother plant of all selected genotype that confirmed the clonal uniformity.

Keywords: Musa spp., Micropropagation, Plant harmones, Genetic fidelity, Molecular markers

1. INTRODUCTION

Banana is a monocotyledonous, perennial stooling herb contains a wide range of triploid (2n=3x=33) cultivars that evolved from interspecific and intraspecific hybrids of the two wild diploid species M. acuminate Colla and M. Balbisisana Colla [1]. Edible bananas (Musa acuminata) are the major staple fruit and vegetable crops for urban consumers in the tropical and subtropical countries. It is an important source of rural income [2]. World banana production is around 103,000 thousand tons annually [3]. India gain top slot by producing the manifold of bananas with 27,575 thousand tonnes followed by China with 12,075 thousand tonnes. Tamil Nadu is the top banana producing state of India producing 8016.35 thousand tonnes, followed by Maharashtra 4,100 thousand tonnes [4]. Banana has multiple uses so popularly known as Kalpataru [2][5]. Grand Naine an introduction from France is the most wide spreading commercial cultivar of India. It grows to 5-7 feet and solid green in colour. It is a tall mutant derived from Dwarf Cavendish and is grown on huge plantations in Central America, resistant to blossom end rot that can occur on Dwarf Cavendish. It is one of the most important commercial cultivar worldwide due to its resistance to wind throw and

production of large bunches and fingers despite its relatively small stature compared to other cultivars of banana. Plant character resembles with Dwarf Cavendish except for its robustness, finger of bigger size and heavy bunches. It bears bunches of weight 25-30 kg with uniform long fingers. It is very attractive for its landscaping potential and good wind resistance [2].

The transportation of bulky suckers for commercial planting is not economical, offers introduction of deadly diseases of banana. Nevertheless in the banana production system the non-availability of diseases-free, true to type planting material, low fertility due to triploidy, slow propagation, long time span from one generation to the next and the soil borne diseases transmitted through suckers and bunchy top diseases are the major constraints. Tissue culture offers methods for faster multiplication of superior clones and production of diseases free propagules through meristem shoot tip culture. In vitro propagation has many advantages such as higher rates of multiplying clean (pest and disease free) planting material and the small amount of space required to multiply large number of plants [5]. Micropropagation [6][7][8][9][10] has major supporting role in biotechnology for rapid multiplication of rare, exotic, endangered species and genetically engineered plants, large production of superior propagules and conservation of economically important ornamental, horticultural, plantation crops and medicinal plants. Genetic fidelity is the maintenance of the genetic constitution of a particular clone through its life span. Clonal uniformity confirming genetic fidelity in all micropropagated plants is an important part of quality assurance for commercial production. Undesirable and non-uniformity (somaclonal variants) in plants can result from stressful condition including the use of plant hormones during the propagation process, which can cause production losses and consequently will affect the attributes of plants production via in vitro technology [2].

Currently there are numerous methods available which detecting the genetic fidelity of micropropagated plants [11]. Molecular marker like SSR and ISSR are a powerful technique for identification of genetic variation [12][13][14][15]. DNA based molecular markers are anchored and ubiquitous to most of the living organisms and have become an important tool to check the genetic uniformity and true-to-type nature of the micropropagated plants [5]. Molecular markers suitable for generating DNA profiles have proved to be an effective tool in assessing the genetic stability of regenerated plants. Therefore, on this context the present investigation was tackled with following objectives.

a) Micropropagation of banana genotype Grand Naine (G-9)

b) Identification of molecular markers for deciphering genetic fidelity in micropropagated banana genotype Grand Naine (G-9)

The present investigation wasset-in-motion with the premise that, micropropagation of banana will be effective method for production of genetically stable (true to type) plants on a large scale. Furthermore ISSR and SSR markers can be employed for testing of genetic fidelity of banana cv. Grand Naine.

2. MATERIAL AND METHODS

Plant material

In present investigation, different banana genotypes Grand naine (G-9), Basrai and Shrimantee were pre owed collected from Vidarbh region, Maharashtra state of India. Sword suckers of banana were used as explant.

Culture medium

In the present study for establishment of explants, Murashige and Skoog (MS) media with full strength were pre owed in combination with plant hormone 5 mg/l BAP and 39 mg/l AdSO4. Furthermore for rooting M.S. media fortified with growth harmones viz., 2 mg/l BAP, 1mg/l IBA and 200mg/l activated charcoal were used under present investigation (Demissie A. G., 2013). The pH of the both media was adjusted to 5.7– 5.8 with 0.1 N HCl or NaOH before adding 0.8% agar and autoclaved at 121°C for 15 minutes.

Molecular marker used for fidelity testing

Different ISSR and SSR primers were used in present investigation viz., UBC 866, UBC 825, ISSCR 8, ISSCR 4, UBC 816, P-16, P-17, UBC 840, IG-19, UBC 834, UBC 812, UBC 841 (ISSR), Mb1–5, Mb1-30, Mb1-50, Mb1-52, Mb1-69, Mb1-113, Mb1-139, Mb1-146, Mb1-148 and Mb1-49-2 (SSR).

Micropropagation of banana genotype Grand Naine

Sword suckers of banana genotypes were taken from the mother nursery block as shown in fig 1 (A). These suckers were pretreated with different surface sterilizers such as 0.1% bavistin, 0.1% streptocycline, 0.1% HgCl2 as shown in fig 1 (B). The surface sterilized and aseptically excised explants (as shown in fig 1C & D) were transferred on the bottles containing medium prepared as per different treatments as shown in fig 1 E. Culture was incubated at 25 ± 2°C in an air-conditioned culture room with a light intensity of 2000-3000 lux by cool white florescent tubes. The light/dark cycles of photoperiod were maintained as 16/8 hrs daily. Subculturing was done by trimming tip of emerging axillary bud and removal of dead tissue at base of explant. Proliferated buds were transferred to different rooting medium. Rooted plantlets were thoroughly washed to free from media. Explants were kept in 0.1% bavistin antifungal solution for 10-15 minutes. For primary hardening, plantlets were transferred to trays containing sterilized substrate cocopit and soil rite maintained in shade house under controlled condition. For secondary hardening, Plantlets were transferred to poly bags containing sand and soil (1:1) substrates. Plants were hardened by gradually increasing light intensity and reducing relative humidity (40-30%).

Statistical analysis

Statistical analysis was done by calculating the recorded data into 10 plantlets per three replications using completely randomized design (CRD).

DNA isolation

DNA isolation of mother plant (leaf) and tissue culture raised banana planlets was done using CTAB extraction method [16] with certain modifications. 0.2 gm of tissue sample was weighed and macerated in mortar pestle. Further 1 ml of pre-warmed CTAB extraction buffer was added into it. 1.5 ml microcentrifuge tubes were inverted for 2-3 times and incubated in water bath for 1 hour at 65°C.The tubes were centrifuged at 10000 rpm for 10 min at 4°C. Supernatant was taken and equal volume of chloroform isoamlyalcohol (24:1) was added in the supernatant followed by manual shaking. Centrifugation was carried out at 10000 rpm for 10 min at 4°C.Then aqueous phase was removed and taken in another centrifuge tube without disturbing the interphase. Equal volume of prechilled isopropanol was added.Centrifugation was carried out at 10,000 rpm for 15 min at 4°C.The supernatant was removed and the pellet was washed with 70% ethanol. Pellet was air dried for 30 to 45 min

and dissolved in TE buffer stored at 4°C for future use. Afterword DNA confirmation if RNA trace was seen then RNAse treatment was given to the sample in 10:1 ratio at 37°C for 1 hr.

DNA quantification

The 0.8 % agarose gel was used for DNA band separation. Quantity and quality of total DNA isolated from target tissue samples were assessed by spectrophotometer or nanodrop. The amount of DNA was calculated by using the formula given below,

$$DNA\left(\frac{ng}{\mu l}\right) = \frac{A260\ X50X\ dilutionfactor}{1000}$$

PCR amplification for ISSR and SSR markers

Synthesized primers were primarily dissolved in deionized water to get final stock concentration 100 pmol/ul. These stocks were diluted to pmol/ul in ratio 1:9. Different PCR components for preparation of PCR reaction mixture 10 X PCR buffer 2.5ml, 2 mM dNTPs 2.0 ml, 1U Taq DNA polymerase 0.2 ml, 25mM MgCl2 1.0 ml, Primer 0.8 ml, Genomic DNA/ Template 1.0 ml, Nuclease free water 12.5 ml with total 20 ml reaction mixture. The PCR was performed in Master cycler (Eppendorf, Germany) using a program for the ISSR and SSR primers. Following PCR program was perform initial denaturation 94°C for 4 min, denaturation 94°C for 1 min, annealing 45-65°C 1 min, extension 72°C for 2 min with 35 repeated cycles followed by final extension 72°C for 10 min. PCR product analysis was carried out on PAGE (8 % Polyacrylamide gel electrophoresis) followed by silver staining [17].

Data analysis

The gel image was analyzed using software SYNGENE (gel documentation unit). Clear, reproducible and scorable band considered for analysis.

3. RESULTS AND DISCUSSION

The present investigation was performed to appraise the genetic fidelity of different micropropagated banana genotypes comparing with mother plant during different stages of subculture.

Micropropagation of banana genotype Grand Naine

Establishment of contamination free cultures is the first step in development of the regeneration protocol for any plant species. To avoid contamination during initiation, antibiotic viz., rifampicin (3 ml/l) and cefotaxime (2.5 ml/l) were added in initiation medium as shown in fig 2 A. Inoculated bottles were kept for incubation in growth room under 16 hrs photoperiods and temperature was maintained at 25 ± 2°C. The banana shoot-tip explants were creamy white in colour and turned brownish in color after 6 to 8 days as shown in fig 2 B. The leaf sheaths were turned green after 10 to 15 days of inoculation as shown in fig 2 C. Subculture was carried out when proliferation of shoots from the explants was done. Stimulation of axillary buds to develop in to shoot took place during initiation of multiplication stage. Initial sub culturing was done when the explants exhibited unwhorling of leaf sheaths after 21st day of inoculation as shown in fig 2 D. After 21st day of inoculation subculture was carried out by trimming basal dead tissues and brown portion of explants. Repeated

subculture was carried out to control contamination, media depletion and browning. The shoots which survived in initiation culture were subculture after 21th day. At second subculture which was carried after 50th day of inoculation, the multiple shoot clumps were separated and inoculated on separate bottles. The observation recorded after 50th days of inoculation revealed that the central meristem produces clusters of proliferating buds and one to three axillary buds get regenerated from basal parts of explant around central apical meristem as shown in fig 2 F. At 50th days of inoculation, the second subculture was carried out to separate all multiple shoots as shown in fig 2 H. Proliferated multiples were separated and transferred into rooting medium containing different media as shown in fig 2 I. 90 % and 75% plants were successfully established during primary hardening (as shown in fig 2 J) in controlled as well as in natural field condition (as shown in fig 2 K)[5].

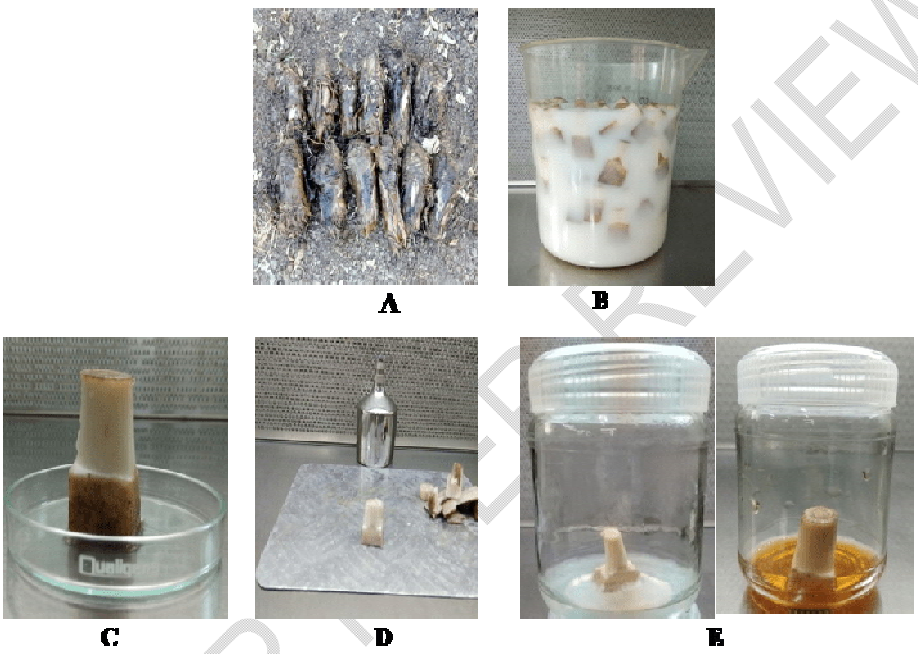


Fig. 1 Preparation of explant and inoculation of shoot tip from suckers of banana cv. Grand Naine A) Collection of explants B) Pretreatment C) Explant D) Final cut under LAF cabinet E) Inoculation of explant in solid & liquid medium

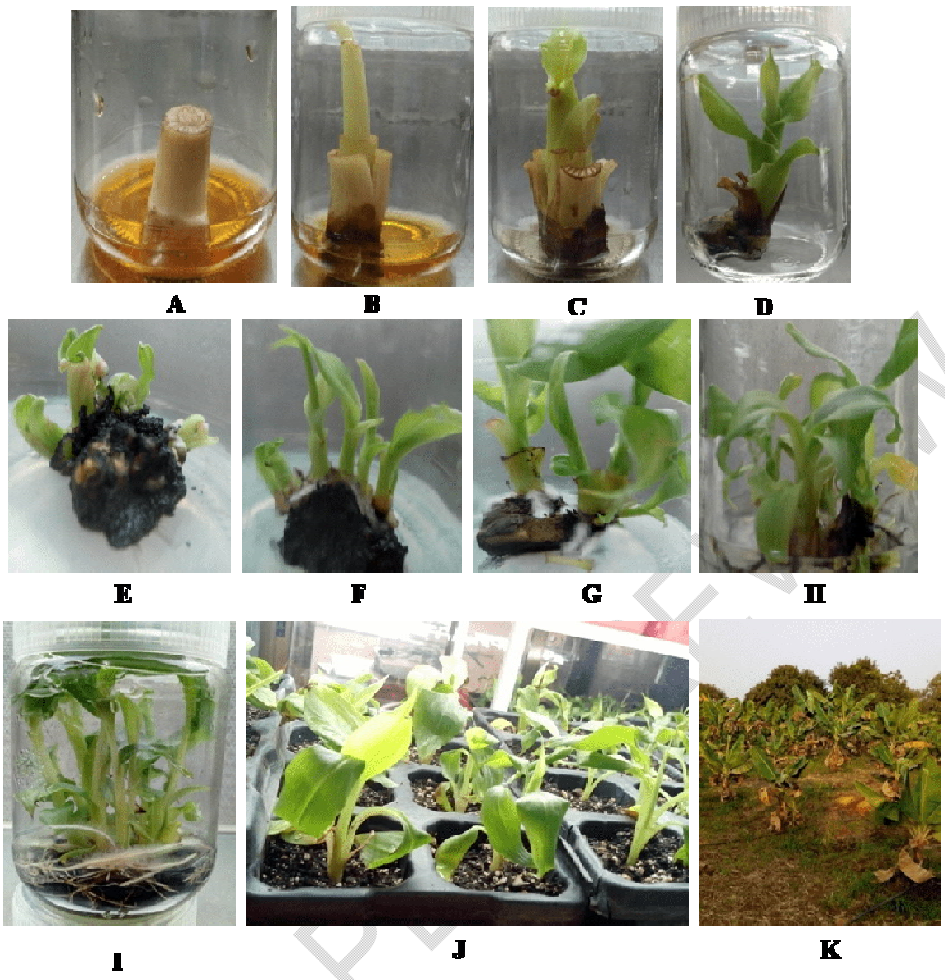


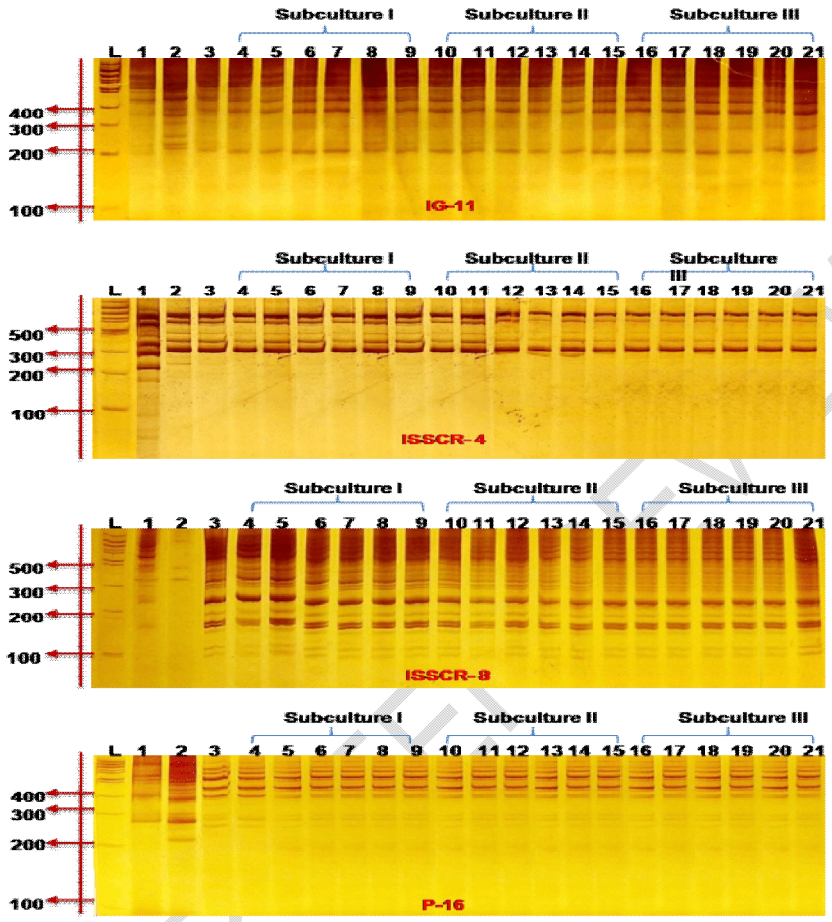
Fig. 2 Initiation, culture establishment & multiplication of banana cv. Grand Naine A) On 1st day B) After 8th days C) After 15th days D) After 21st days, Also different stages of multiplication after 8th days (E), 15th days (F), 21st days (G) & 30th days (H) days on multiplication medium & I) Rooting J) Primary hardening K) Secondary hardening

Genetic fidelity of banana genotypes Shrimantee, Basrai and Grand Naine

Total 26 (16 ISSR and 10 SSR) [3][18] [19] molecular markers were screened for fidelity testing in Grand Naine and genetic identity of different genotypes of banana Shrimantee, Basrai and Grand Naine. Total twenty (14 ISSR and 6 SSR) primers were produced scorable and clear bands with product size ranging from 100-2000 bp. The 20 primers (ISSR and SSR) were produced totally 146 clear, reproducible and scorable bands. The primers ISSCR-8 and IG-19 were amplified the highest (12) number of bands. Whereas primers Mb1-05 and P-17 were

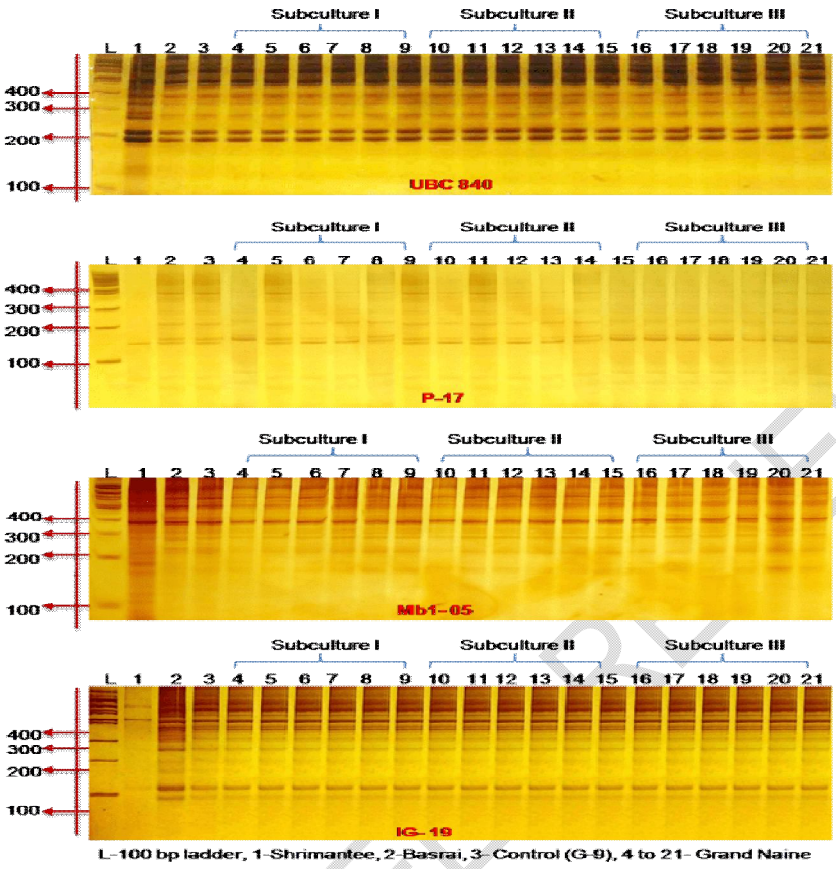
amplified the lowest (3) number of bands. The number of bands for each primer varied from 3 (Mb1-05 and P-17) to 12 (ISSCR-8 and IG-19) with an average 7.3 bands per primer [12][13][20]. Also for Grand Naine genotype average 4.45 bands per primer, for Shrimantee

genotype average 3.05 bands per primer and for Basrai genotype average 4.9 bands per primer were produced



L-100 bp ladder, 1-Shrimantee, 2-Basrai, 3- Control (G-9), 4 to 21- Grand Naine

Fig. 3 Amplification pattern of banana genotypes using primer IG-11, ISSCR-4, ISSR-8 and P-16



L-100 bp ladder, 1-Shrimantee, 2-Basrai, 3- Control (G-9), 4 to 21- Grand Naine

Fig. 4 Amplification pattern of banana genotypes using primer UBC 840, P-17, MB1-05 & IG-19

4. CONCLUSION

During micropropagation, six randomly selected healthy tissue cultured plants were taken after three subculturing for genetic fidelity testing using ISSR and SSR markers. The banding pattern of these randomly selected plants was monomorphic with mother plant of genotype Grand Naine.

Screening of random selected samples of banana cultivars produces genetic stable, monomorphic and uniform clones. There were no changes found in the banding pattern of tissue culture plants so in vitro multiplication is the safest mode for multiplying of true to type plants. So all micropropagated banana cv. Grand Naine showed uniformity from that we can concluded that they are clones of each others. Molecular markers were utilized successfully in genetic fidelity of micropropagated plantlets of banana[2][14][21]. Therefore used molecular markers were found very effective in differentiating the Grand Naine from Shrimantee and Basrai and genetic fidelity testing of Grand Naine [22].

Further study needed for assuring the somaclonal variation in tissue culture raised plants upto 5-6 subculture.

CONSENT (WHERE EVER APPLICABLE)

No, It is not applicable.

ETHICAL APPROVAL

It is not applicable

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