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Full Length Research Paper

In vitro callus induction and plant regeneration from mature seed embryo and young shoots in a giant sympodial bamboo, *Dendrocalamus farinosus* (Keng et Keng f.) Chia et H.L. Fung

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The method for callus induction, adventitious shoot induction and plant regeneration derived from mature embryos of the seed and young shoots in *Dendrocalamus farinosus* was examined. For young shoots, the lowest callus induction frequency (21.0 to 29.7%) was obtained and the induced callus became brown and perished after two weeks. For mature embryos of the seed, an efficient protocol for callus induction, adventitious shoot induction and plant regeneration was developed. The best callus induction medium for mature embryos was observed to be Murashige and Skoog (MS) supplemented with 2.0 mg l^{-1} 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in combination with 0.2 mg l^{-1} kinetin (Kn) plus 0.4 mg l^{-1} indole-3-butyric acid (IBA). Callus induction frequency was 95%. The highest adventitious shoot induction frequency (91.2 ± 2.18%) was obtained on MS medium supplemented with 2.5 mg l^{-1} kn plus 0.5 mg l^{-1} indole-3-acetic acid (IAA). The regenerated adventitious shoots were rooted *in vitro* on MS medium with 0.4 mg l^{-1} IBA plus 0.25 mg l^{-1} IAA. Rooted plantlets successfully acclimatized to the greenhouse and 90.1% survived after being transplanted to plastic pots (measuring 30 cm in diameter) with garden soil.

Key words: Callus culture, plant regeneration, making-pulp use, *Dendrocalamus farinosus.*

INTRODUCTION

Bamboo is the common term applied to a broad group (comprising 1,250 species) of large woody grass, with height ranging from 10 cm to 40 m. Currently used daily by approximately 2.5 billion people in the Asian region, mostly for fiber and food, bamboo may have potential as a bioenergy or fiber crop for niche markets (Scurlocka et al., 2000). Short rotation, annual marketability of culms and immediate returns has made the bamboo a suitable species for plantation and agroforestry (Christanty et al., 1997). Bamboo fibers are relatively long (measuring 1.5 to 3.2 mm) and thus, ideal for paper production (El Bassam, 1998). Incidentally, paper production in China dates back to 2000 years ago (Cui et al., 2010).

Native to Sichuan province of China, *Dendrocalamus farinosus* is an important sympodial bamboo species with more cold tolerance and high yield. It produces good-quality wood for pulping. It occupies an area of 27,000 ha in Sichuan province (Wang et al., 2009). Genetic improvement is important for the development of the said species to increase quality and yield. However, routine genetic improvement protocols are limited in *D. farinosus*, mainly because of its long flowering cycle. Therefore, *in vitro* callus induction and plant regeneration approach by transferring foreign gene are important for its improvement.

Compared with conventional breeding strategies, genetic engineering is a more feasible tool for producing plants by reducing lignin content and modifying lignin components. Successful genetic transformation by modifying lignin content or lignin component genes into tree species has been reported (Hu et al., 1999; Wagner

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et al., 2007; Coleman et al., 2008; Stewart et al., 2009). In recent years, micropropagation of bamboo (Ramanayake and Wanniarachchi, 2003; Ramanayake et al., 2006; Ramanayake et al., 2008) and cloning of certain genes, such as 4-coumarate-coenzyme A ligase gene (EU327341) from *Neosinocalamus affinis* (Hu et al., 2009) and catechol-O-methyltransferase gene from *Bambusa oldhamii* (Li et al., 2007), were mainly reported. At present, there are no reports describing the successful genetic transformation of foreign gene transfer to bamboo. Therefore, callus induction and plant regeneration of bamboo is a prerequisite for genetic modification via *Agrobacterium*-mediated transformation.

From what is known, studies on *D. farinosus* mainly focus on its genetic diversities (Jiang et al., 2008). There are no reports describing the *in vitro* callus and plant regeneration of *D. farinosus*. This paper reports for the first time, a protocol for callus induction and plant regeneration derived from mature seed embryo in a giant sympodial bamboo; *D. farinosus*. It is envisioned to provide sound experimental foundation for future selection of somaclonal variation and genetic transformation.

MATERIALS AND METHODS

Plant material and surface-decontamination

Mature seeds and young shoots (20 cm in height) of *D. farinosus* were obtained from the parent plants in 2009 at Luzhou City of Sichuan province, China. Healthy seeds were rinsed in running water for 5 min. Subsequently, these were soaked in water for 24 h at 25°C and washed in running water for 3 min. The surface was sterilized sequentially for 30 s in 70% (v/v) ethanol, rinsed thrice in sterile distilled water, stirred for 30 min in 0.1% $HgCl_2$ (v/v) and finally, rinsed six times in sterile distilled water.

Similarly, the healthy young shoot-top (the length of the young shoot-top used was 3 cm) were rinsed in running water for 5 min. The surface was sterilized sequentially for 30 s in 70% (v/v) ethanol, rinsed thrice in sterile distilled water, stirred for 30 min in 0.1% $HgCl_2$ (v/v) and finally, rinsed six times in sterile distilled water.

Callus induction and subculture

Murashige and Skoog (MS) (Murashige and Skoog, 1962) and woody plant medium (WPM) (Lloyd and McCown, 1980) were used as the basic culture media. The types and concentrations of plant growth regulators used were chosen according to previous works in wheat (Zeng, 1988; Li et al., 1996). Mature seed embryos and the section (Its thickness was 1 mm) of the young shoot-top in *D. farinosus* were cultured in four different culture media: MS medium supplemented with 2.0 mg l^{-1} 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) in combination with 0.2 mg l^{-1} kinetin (Kn) and 0.4 mg l^{-1} indole-3-butyric acid (IBA), MS medium supplemented with 2.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) in combination with 0.5 mg l^{-1} kn and 0.1 mg l^{-1} indole-3-acetic acid (IAA), woody plant medium (WPM) supplemented with 2.0 mg l^{-1} 2,4,5-T in combination with 0.2 mg l^{-1} kn and 0.4 mg l^{-1} IBA and WPM medium supplemented with 2.0 mg l^{-1} 2,4-D in combination with 0.5 mg l^{-1} kn and 0.1 mg l^{-1} IAA. All culture media utilized in the study were supplemented with 3.0% sucrose and 0.7% agar powder and adjusted to pH 5.8 before autoclaving at 1.0 kg cm^{-2} at 121°C for 20 min. For callus induction, mature embryos were incubated for seven days at 25 ± 1°C in the dark for dedifferentiation, followed by incubation at 25 ± 1°C under a 12 h photoperiod (80 µmol m^{-2} s^{-1}) for three weeks. After initial callus induction, calluses were sub-cultured in fresh media every four weeks to observe the effect of 2,4,5-T and 2,4-D in MS and WPM on callus induction. Fresh callus weight from 50 mature seed embryos was determined at 112 days old.

Adventitious shoot induction from calluses

The types and concentrations of plant growth regulators and the basis medium used for adventitious shoot induction from calluses were chosen according to previous works in wheat (Zeng, 1988; Li et al., 1996). Light yellow loose fragile calluses were transferred to the MS medium supplemented with kn at different levels (1.5 and 2.5 mg l^{-1}) plus 0.5 mg l^{-1} IAA to induce shoot regeneration at 25 ± 1°C under a 12 h photoperiod (80 µmol m^{-2} s^{-1}) for two weeks. After adventitious shoot regeneration induction, they were sub-cultured in fresh media every four weeks for shoot elongation.

Rooting of adventitious shoots

Elongated adventitious shoots measuring 3.0 to 4.0 cm in length were transferred for rooting to the MS medium supplemented with 0.4 mg l^{-1} IBA plus IAA at different levels (0, 0.25 and 0.5 mg l^{-1}) at 25 ± 1°C under a 12 h photoperiod (80 µmol m^{-2} s^{-1}) for two weeks.

Acclimatization of rooted plants and transplanting

Rooted plantlets were rinsed gently in distilled water to remove any agar from the roots. Plantlets were transplanted into the plastic pots (measuring 15 cm in diameter) containing moist, autoclaved peat moss, vermiculite and garden soil mixture (with a ratio of 2:1:1). Pots were placed in plastic bags to provide high relative humidity. Plantlets in pots were watered every three days with running water. They were allowed to acclimatize over a period of 14 days in the culture room by progressively opening the bag until plantlets were ready for transfer to the greenhouse. After two weeks, plantlets in small pots were transplanted into plastic pots (measuring 30 cm in diameter) containing autoclaved garden soil under natural light. They were cultured for two weeks and the number of surviving transplanted plantlets was recorded.

Statistical analysis

For induction of calluses from mature seed embryos, each treatment used 200 mature embryos. For adventitious shoot regeneration from light yellow loose fragile callus, 40 replicates of the respective calluses masses were inoculated onto MS medium supplemented with 2.0 mg l^{-1} 2,4,5-T in combination with 0.2 mg l^{-1} kn and 0.4 mg l^{-1} IBA; subsequently, they were inoculated onto MS medium supplemented with kn at different levels (1.5 and 2.5 mg l^{-1}) plus 0.5 mg l^{-1} IAA. 40 replicates were used for shoot elongation. For rooting of shoots, 40 replicates were maintained and 100 plantlets were transplanted into plastic pots with garden soil for the survival rate of transplanted plantlets. The experiment was conducted thrice. Data were analyzed using SAS 9.0 software.

RESULTS

Callus induction and formation

After 29 to 35 days, callus initiation from the top of the

Table 1. Effect of plant growth regulator combination and medium type on callus induction (30th day).

Medium composition	Mature embryo			Young shoot	
	Duration of callus induction (day)	Callus formation (%)	Fresh callus weight (g)	Duration of callus induction (day)	Callus formation (%)
WPM + 2,4-D (2.0 mg l^{-1}) + kn (0.5 mg l^{-1}) + IAA (0.1 mg l^{-1})	7	81.5 ± 0.79^b	0.2952 ± 0.18^c	35	21.0 ± 1.24^a
MS + 2,4-D (2.0 mg l^{-1}) + kn (0.5 mg l^{-1}) + IAA (0.1 mg l^{-1})	7	82.8 ± 0.87^b	0.6038 ± 0.15^b	30	28.6 ± 1.13^a
WPM + 2,4,5-T (2.0 mg l^{-1}) + kn (0.2 mg l^{-1}) + IBA (0.4 mg l^{-1})	7	92.6 ± 0.76^a	0.4026 ± 0.13^c	34	22.3 ± 0.97^a
MS + 2,4,5-T (2.0 mg l^{-1}) + kn (0.2 mg l^{-1}) + IBA (0.4 mg l^{-1})	7	96.0 ± 0.95^a	0.8251 ± 0.12^a	29	29.7 ± 1.02^a

Values (% ± S.D.) and values (g ± S.D.) within column followed by the different letters were significantly different at P < 0.05. Fresh callus weight was the average of fresh weight of callus obtained from 50 mature embryos at 112 days. Duration of callus induction' is the time taken for incubation of mature seed embryos or young shoot to callus initiation.

young shoot was observed in the four medium types indicated earlier. Low callus induction frequency (21.0 to 29.7%) was recorded (Table 1). The low proliferating callus turned brown and died after two weeks (Figure 1a). However, for callus induction of mature seed embryo, callus formation began after seven days (Figure 1b) in the four medium types. Callus induction was associated with medium type under the same condition of plant growth regulator combination (Table 1). Callus induction frequency in MS medium was higher than that in woody plant medium (WPM). Fast growing callus formation and proliferation in mature embryos were observed in MS as the basic culture medium. Therefore, compared with WPM, MS proved to be a more feasible basic culture medium for inducing callus formation derived from mature embryos of *D. farinosus*. High callus induction frequency (96.0%), fastest growing callus formation and high proliferating light yellow loose friable granule callus (Figure 1c; Table 1) were observed on the MS medium supplemented with 2.0 mg l^{-1} 2,4,5-T in combination with 0.2 mg l^{-1} kn and 0.4 mg l^{-1} IBA. In this study, mature seed embryo possessed greater callus induction potential compared with young shoot.

Adventitious shoot induction and elongation

It was found that the light yellow loose friable granule callus was cultured for 50 days in the medium (MS supplemented with 2.0 mg l^{-1} 2,4,5-T in combination with 0.2 mg l^{-1} kn and 0.4 mg l^{-1} IBA) used for callus induction; the calluses became more greenish and appeared highly competent for shoot bud initiation and rooting (Figure 1d). They were then regenerated to plantlets, but the rate of regenerating plantlet was low (Table 2).

Meanwhile, light yellow loose friable granule calluses were transferred to MS medium supplemented with kn at different levels; 2.5 and 1.5 mg l^{-1} plus 0.5 mg l^{-1} IAA to induce shoot regeneration. After two weeks, the rate of shoot regeneration and shoot length for the 2.5 mg l^{-1} level were higher than those for 1.5 mg l^{-1} level (Table 2). 0.02% of the albino shoot bud derived from thecallus with purple spot (Figure 1g, h) was observed. In this study, MS medium supplemented with 2.5 mg l^{-1} kn plus 0.5 mg l^{-1} IAA performed well for adventitious shoot regeneration (Figure 1e, f), elongation and shoot proliferation (Figure 1i and Table 2).

Shoot rooting and transplanting

Rooting of regenerated adventitious shoots did not occur in the shoot induction MS medium supplemented with 2.5 mg l^{-1} kn plus 0.5 mg l^{-1} IAA even after a culture period of over six weeks. Therefore, regenerated shoots were transferred to MS supplemented with 0.4 mg l^{-1} IBA plus IAA at different levels (0, 0.25 and 0.50 mg l^{-1}) to induce rooting for two weeks. High rooting percentages (71.4 to 95.6%) were achieved using MS supplemented with IBA plus IAA. The highest rooting (95.6%) and mean number of roots per shoot (6.2 ± 1.34) (Figure 1j and Table 3) were obtained from the MS medium with 0.4 mg l^{-1} IBA plus 0.25 mg l^{-1} IAA. The MS medium with 0.4 mg l^{-1} IBA plus 0.50 mg l^{-1} IAA induced 87.8% of rooting in the regenerated shoot, but the number of roots per shoot was lower (2.9 ± 1.18). Only 71.4% of rooting was observed in the MS medium with 0.4 mg l^{-1} IBA. In this study, MS medium with 0.4 mg l^{-1} IBA plus 0.25 mg l^{-1} IAA was observed to be best for rooting.

100 rooted plantlets with well-developed roots were transplanted to plastic pots for acclimatization testing (Figure 1k) for two weeks. Subsequently, plantlets in small pots were transferred to plastic pots of 30 cm in diameter (Figure 1l). After two weeks, survival rate of

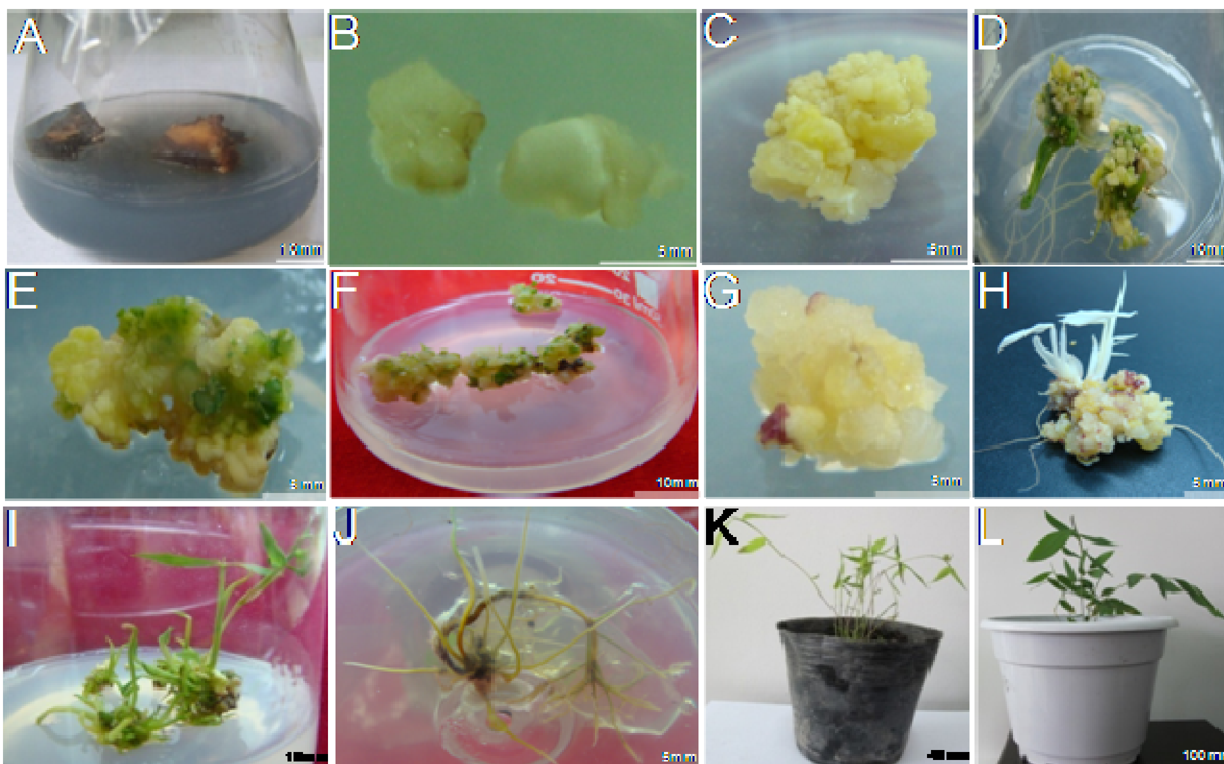

Figure 1. Callus induction and plant regeneration from mature seed embryos and young shoots of *D. farinosus*. (A) Brown callus from young shoots after two weeks of callus initiation; (B) callus initiation from mature seed embryos cultured in dark on MS supplemented with 2.0 mg l^{-1} 2,4,5-T in combination with 0.2 mg l^{-1} kn and 0.4 mg l^{-1} IBA after 7 days; (C) light yellow loose friable granule callus from mature seed embryos on MS supplemented with 2.0 mg l^{-1} 2,4,5-T in combination with 0.2 mg l^{-1} kn and 0.4 mg l^{-1} IBA after 30 days; (D) adventitious shoot initiation and root formation from the same callus derived from mature seed embryos on MS supplemented with 2.0 mg l^{-1} 2,4,5-T in combination with 0.2 mg l^{-1} kn and 0.4 mg l^{-1} IBA after 50 days; (E, F) adventitious shoot induction on MS supplemented with 2.5 mg l^{-1} kn plus 0.5 mg l^{-1} IAA after two weeks; (G) callus with purple spot; (H) albino shoot bud derived from callus with purple spot on MS supplemented with 2.5 mg l^{-1} kn plus 0.5 mg l^{-1} IAA; (I) adventitious shoot elongation on MS supplemented with 2.5 mg l^{-1} kn plus 0.5 mg l^{-1} IAA after four weeks; (J) rooting of shoots on MS supplemented with 0.4 mg l^{-1} IBA plus 0.25 mg l^{-1} IAA after two weeks; (K) normal growth of potted plantlet in culture room after 14 days; (L) regenerated plant transplanted to plastic pot (30 cm in diameter) with garden soil after two months.

transplanting plantlets was recorded at 90.1%.

DISCUSSION

Callus induction

Callus can be obtained from various bamboo tissues using medium containing auxin, such as 2,4-D (Chang, 1991). However, for giant sympodial bamboo species with excellent features for pulping such as *D. farinosus*, callus induction and plant regeneration are major problems encountered during the improvement process by inducing somaclonal variation and transferring of foreign gene (Wang et al., 2009). In this study, a protocol for callus induction and plant regeneration of *D. farinosus* was reported. In this study, it was found that the MS medium containing 2,4,5-T (2.0 mg l^{-1}) in combination with kn (0.2 mg l^{-1}) and IBA (0.4 mg l^{-1}) was superior to the MS containing 2,4-D (2.0 mg l^{-1}) in combination with kn (0.5 mg l^{-1}) and IAA (0.1 mg l^{-1}) for high proliferation of light yellow loose friable granule callus with high potential for plantlet regeneration (Figure 1d and Table 1). However, a number of studies revealed that, the MS medium with 2,4-D in combination with kn or NAA or BA was used for inducing callus of bamboo (Yeh and Chang, 1986; Ramanayake and Wanniarachchi, 2003; Godbole et al., 2002). For *Bambusa oldhamii Munro*, embryogenic callus was initiated from explants maintained on Murashige and Skoog's medium supplemented with 3 mg l^{-1} 2,4-D, 2 mg l^{-1} kn (Yeh and Chang, 1986). For *D. giganteus* Wall. ex Munro, callus induced and proliferated in MS medium containing 33.9 µM (7.5 mg l^{-1}) 2,4-D and 16.1 µM (3.0 mg l^{-1}) NAA formed nodular callus with low potential for plantlet regeneration (Ramanayake and Wanniarachchi, 2003). Murashige and Skoog medium with BA and 2,4-D (1.0 mg/l each) was essential for culture establishment and callusing of *Dendrocalamus*

Table 2. Effect of plant growth regulator combination on shoot regeneration and elongation (28th day).

Plant growth regulator (mg l^{-1})				Shoot formation (%)	Mean number of shoots/unit callus	Mean shoot length (cm)
2,4,5-T	kn	IBA	IAA			
2.0	0.2	0.4		45.8 ± 2.06^c	5.78 ± 0.61^a	3.98 ± 1.03^b
	1.5		0.5	64.2 ± 3.62^b	2.21 ± 0.37^b	3.29 ± 1.15^b
	2.5		0.5	91.2 ± 2.18^a	6.34 ± 0.56^a	5.36 ± 1.19^a

Values represent mean ± standard error of 40 replicates per treatment in three repeated experiments. Means with the same letter were not significantly different (P = 0.05). Values (% ± S.D.) within column followed by the different letters were significantly different at P < 0.05.

Table 3. Effect of plant growth regulator combination on root formation in vitro of micro shoots (28th day).

Treatment IBA + IAA (mg l^{-1})	Rooting (%)	Mean number of root
Control	0	0
0.4 + 0	71.4 ± 0.2^b	2.1 ± 1.21^b
0.4 + 0.25	95.6 ± 0.5^a	6.2 ± 1.34^a
0.4 + 0.50	87.8 ± 0.2^a	2.9 ± 1.18^b

Values represent mean ± standard error of 40 replicates per treatment in three repeated experiments. Means with the same letter were not significantly different (P = 0.05). Values (% ± S.D.) within column followed by the different letters were significantly different at P < 0.05.

hamiltonii (Godbole et al., 2002).

Plant regeneration

For shoot induction, multiple shoots of *Thamnocalamus spathiflorus* (Trin.) Munro was induced in MS medium supplemented with 5.0 µM 6-benzylaminopurine (BAP) and 1.0 µM indole-3-butyric acid (IBA) (Bag et al., 2000). In this study, MS medium supplemented with 2.5 mg l^{-1} kn plus 0.5 mg l^{-1} IAA worked favorably for adventitious shoot regeneration, elongation and shoot proliferation of *D. farinosus* (Table 2). In addition, it was found that the regeneration plantlets may be also obtained from the medium (MS supplemented with 2.0 mg l^{-1} 2,4,5-T in combination with 0.2 mg l^{-1} kn and 0.4 mg l^{-1} IBA) used for callus induction (Table 2).

For shoot rooting, auxin IBA has been reportedly useful for inducing rooting in a variety of plants (Hammatt and Ridout, 1992; Perez-Parron et al., 1994; Ramanayake et al., 2006, 2008; Faisal et al., 2007; Mishra et al., 2008). For *Tylophora indica*, Faisal et al. (2007) reported that IBA was superior to IAA and NAA in inducing rooting. Ramanayake et al. (2006) reported that, a rooting medium with IBA at 3 mg l^{-1} was optimum for rooting induction and MS was used for rooting in *Bambusa vulgaris* 'Striata'. In this study, the optimal rooting medium was MS medium with 0.4 mg l^{-1} IBA plus 0.25 mg l^{-1} IAA.

Conclusions

This study developed an inducing callus, adventitious shoot regeneration, rooting, acclimatization and transplanting protocol from mature embryos of seed in *D. farinosus*. It is a rapid system, requiring merely 16 weeks from initiation of callus to transplanting of plantlets to greenhouse. This protocol will be useful for conducting genetic transformation, inducing somaclonal variation and inducing mutagenesis in *D. farinosus*.

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