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Gibberellins-Producing Rhizobacteria Increase Endogenous Gibberellins Content and Promote Growth of Red Peppers

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The growth of red pepper plants was enhanced by treatment with the rhizobacterium, *Bacillus cereus* MJ-1. Red pepper shoots showed a 1.38-fold increase in fresh weight (fw) and roots showed a 1.28-fold fw gain. This plant growth-promoting rhizobacterium (PGPR) has been reported to produce gibberellins (GAs). Other GAs-producing rhizobacteria, *Bacillus macroides* CJ-29 and *Bacillus pumilus* CJ-69, also enhanced the fw of the plants. They were less effective than *B. cereus* MJ-1, though. The endogenous GAs content of pepper shoots inoculated with MJ-1 was also higher than in shoots inoculated with CJ-29 or CJ-69. When inoculated with MJ-1, bacterial colonization rate of the roots was higher than that of roots inoculated with CJ-29 or CJ-69. These results support the idea that the plant growth-promoting effect of the bacteria also positively related with the efficiency of root colonization by the bacteria. In addition, we identified the major endogenous GAs of the red pepper as originating from both the early C-13 hydroxylation and the early non C-13 hydroxylation pathways, with the latter being the predominant pathway of GA biosynthesis in red pepper shoots.

Key words: growth-promoting interaction between rhizobacteria and plant, rhizobacteria producing gibberellins, root colonization

A large number of different microorganisms, mostly bacteria, are commonly found in the soil. Certain soil bacteria that interact specifically with plant roots in the rhizosphere have the potential to directly increase plant growth. They do this by releasing phytohormones, fixing N_2 in the rhizosphere, solubilizing nutrients like phosphorate, promoting mycorrhizal function in roots, and regulating ethylene production in roots. Beneficial soil bacteria are usually referred to as plant growth-promoting rhizobacteria or PGPR (Kloepper, 1993; Jeon *et al.*, 2003; Park *et al.*, 2005).

Gibberellins (GAs) are a group of phytohormones that influence many developmental process in higher plants, including seed germination, stem elongation, flowering, and fruit setting (Hedden and Kamiya, 1997). To date, 136 GAs from higher plants (128 species), 28 GAs from fungi (7 species), and only 4 GAs (GA_1, GA_3, GA_4, and GA_{20}) from bacteria (7 species) have been identified (MacMillan, 2002). Plant growth promotion by PGPR species that produce GAs has been previously reported (Atzhorn *et al.*, 1988; Bastian *et al.*, 1998; Gutierrez-Manero *et al.*, 2001).

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In previous studies, we reported the promotion of growth in red peppers, an important culinary plant, by rhizobacteria known to produce GAs (Joo *et al.*, 2004). To optimize growth-promotion interaction between the PGPR and plants, it is important to discover exactly how each rhizobacterium exerts its effect on plants. It is also necessary to know whether the effect is altered by various environmental factors, including the presence of other microorganisms. The experiments reported here were performed to determine the role of GAs in the promotion of red pepper shoot growth. The influence of bacterial inoculation on the endogenous GAs content of red pepper was also examined in an attempt to determine if growth promotion by the PGPR was related to changes in hormone levels.

Materials and Methods

Microorganism and culture conditions

The following PGPR producing GAs were used in this study: *Bacillus cereus* MJ-1, *B. macroides* CJ-29, and *B. pumilus* CJ-69 (Joo *et al.*, 2004). They were cultured under aerobic conditions on a GY medium (1 g glucose, 2.5 g yeast extract, 5 g peptone, 0.5 g NaCl, 15 g agar, 1 l water, pH 7.0) at 37 for 3 days. The bacteria were har-

vested, suspended in 0.1 M NaH_2PO_4/NaOH (pH 7.0), and then adjusted to 10^9 c.f.u./ml for soil treatment.

Growth of red pepper plug seedlings

Red pepper plug seedlings were grown from surface-sterilized seeds (*Capsicum annuum* L.; Seminis Korea, Korea) in a Styrofoam box (20 × 30 × 15 cm) filled with wet potting soil (Seminis Korea, Korea) in a dark chamber for 48 h at 22-25°C. The average relative humidity (RN) of the growth chamber was kept at 75%. After being sown, seedlings were placed in a growth chamber (light period of 15 h, 500 lux at 25°C; dark period of 9 h at 25°C, average RN of 75%) and then transplanted individually to a polycarbonate tray (5.5 × 28 × 55 cm) filled with wet potting soil (40 g soil for each seedling) at the three / four-leaf stage. Each seedling was treated with 1 ml of bacteria diluted with tap water to an estimated cell density of 10^8 c.f.u./ml. In order to perform bacterial co-inoculation tests, cells (10^8 c.f.u./ml) of *Bacillus cereus* MJ-1, *B. macroides* CJ-29, or *B. pumilus* CJ-69 were mixed at a ratio of 1:1 (v/v), treated as described above. The tray was placed in a greenhouse (light period at 22-25°C, dark period at 16-22°C) and watered every three days with tap water. Sixty-seven days after inoculation, the endogenous GAs of the shoots of the red pepper were measured.

Root colonization of the PGPR in red pepper

Six red pepper seedlings were taken at 10, 20, 30, 40, and 67 days after the initial treatment. The roots were washed 10 times with 20 ml of GY medium to remove soil. Each root was transferred into a test tube containing 9 ml of 0.85% saline solution and was vigorously stirred. Then, viable cell counts were performed on a tryptic soy agar medium after the plates were incubated for 24 h at 37°C. Microorganisms were identified by morphology and by analysis of their fatty acid profiles using the Sherlock system according to the recommendations of the manufacturer (MIDI, USA).

Extraction of endogenous GAs in red pepper seedlings

Lyophilized tissue samples were weighed and ground to a fine powder in a mortar and pestle with the aid of acid-washed sea sand. The powdered tissue was extracted with 80% (v/v) methanol. GAs were extracted and partially purified according to the procedure of Lee *et al.* (1998). After extraction with 80% methanol, 20 ng of each internal standard were added to tissue extract. All of the deuterated internal standards used in this step were purchased from Prof. L. N. Mander at Australian National University: [17,17-$^2H_2$] GA_1, GA_3, GA_4, GA_5, GA_7, GA_9, GA_{12}, GA_{15}, GA_{19}, GA_{20}, GA_{24}, GA_{44}, and GA_{53}.

Identification and quantification of endogenous GAs

GAs were separated by a Waters 510 HPLC system equipped with a μBondapak C_{18} column (3.9 × 300 mm; Waters, USA). A linear gradient elution of 28-100% methanol with 1% aqueous acetic acid was used at a flow rate of 1.5 ml per min for 40 min. The HPLC fractions were collected at 1 min intervals.

GC-MS analysis of the GAs was carried out using a HP 5973N mass spectrometer connected to a HP 6890 gas chromatograph (Hewlett-Packard, USA) in accordance with the procedures of Joo *et al.* (2004). The identification of GAs was accomplished by comparing their mass spectra and Kovats retention indices (KRIs) with those of authentic standards (Gaskin and MacMillan, 1991). The GAs were quantified by isotope dilution. This was done by calculating the area ratio of endogenous GAs to the deuterated standard GAs that had been added during the extraction step (Joo *et al.*, 2004).

Statistical analysis

All data was assessed by analysis of variance (ANOVA) using a SAS statistical package (SAS, 1988). Mean comparisons were made using Duncan's multiple range test at $P<0.05$.

Results and Discussion

Plant growth promotion of red pepper plug seedlings by the PGPR

B. cereus MJ-1 significantly promoted the growth of red pepper seedlings by increasing the fresh weight (fw) of the roots 1.38-fold and that of the shoots 1.28-fold (Table 1). Single and co-inoculation with *B. macroides* CJ-29 and *B. pumilus* CJ-69 also enhanced the fresh weight of the roots and shoots of the seedlings. However, the growth-promoting efficiency was less than that of plants singly inoculated or co-inoculated with *B. cereus* MJ-1

Table 1. The growth promotion of red pepper plug seedlings by single and co-inoculation of *Bacillus cereus* MJ-1, *B. macroides* CJ-29, and *B. pumilus* CJ-69

Inocula	Fresh weight (g/plant)	
	Roots	Shoots
Untreated control	0.81 ± 0.13 b	2.46 ± 0.28 a
B. cereus MJ-1	1.06 ± 0.06 a	3.17 ± 0.41 a
B. macroides CJ-29	1.02 ± 0.12 a	3.05 ± 0.24 a
B. pumilus CJ-69	0.96 ± 0.28 ba	2.99 ± 0.33 a
MJ-1 + CJ-29	1.06 ± 0.05 a	3.15 ± 0.38 a
MJ-1 + CJ-69	1.04 ± 0.04 a	3.16 ± 0.27 a
CJ-29 + CJ-69	0.97 ± 0.18 ba	2.99 ± 0.35 a

The data is the mean of 105 seedlings. Each red pepper plug seedling was treated with 1 ml of bacteria diluted with tap water to an estimated cell density of 10^8 c.f.u./ml. Sixty-seven days after the initial treatment, the fresh weight of shoots and roots of red pepper plug seedlings was measured. All values are mean ± SEM, N=105; Values with different characters are significantly different at $P<0.05$.

Table 2. Comparison of endogenous gibberellins (GAs) content by single and co-inoculation of *Bacillus cereus* MJ-1, *B. macroides* CJ-29, and *B. pumilus* CJ-69 in the shoots of red pepper seedlings at sixty-seven days after the initial treatment

GAs	Calculated amounts (ng/g of fresh weight)					
	Inocula					
	Control	MJ-1	CJ-29	CJ-69	MJ-1 + CJ-29	MJ-1 + CJ-69
GA_1	2.1 ± 0.12 d	2.6 ± 0.15 bc	2.5 ± 0.10 dc	2.2 ± 0.01 dc	3.0 ± 0.03 ba	3.1 ± 0.19 a
GA_3	2.6 ± 0.14 ba	2.7 ± 0.15 ba	1.7 ± 0.03 c	1.5 ± 0.14 c	3.0 ± 0.21 a	2.5 ± 0.04 b
GA_4	9.0 ± 0.27 d	11.9 ± 0.07 a	11.5 ± 0.06 ba	10.5 ± 0.28 c	11.0 ± 0.04 bc	11.2 ± 0.17 b
GA_5	2.2 ± 0.11 c	2.1 ± 0.14 c	3.5 ± 0.14 b	4.7 ± 0.26 a	2.3 ± 0.12 c	5.2 ± 0.11a
GA_7	1.7 ± 0.88 c	2.2 ± 0.10 bc	3.4 ± 0.18 ba	2.9 ± 0.10 bc	4.5 ± 0.16 a	3.4 ± 0.10 ba
GA_9	6.9 ± 0.26 d	9.1 ± 0.19 b	8.6 ± 0.15 b	8.0 ± 0.09 c	10.0 ± 0.12 a	10.0 ± 0.12 a
GA_{12}	0.1 ± 0.16 e	4.3 ± 0.11 a	2.1 ± 0.17 d	2.9 ± 0.12 c	3.2 ± 0.10 c	3.7 ± 0.16 b
GA_{15}	2.2 ± 0.04 f	12.1 ± 0.18 a	3.5 ± 0.16 e	4.7 ± 0.13 d	8.3 ± 0.10 b	5.2 ± 0.08 c
GA_{19}	2.7 ± 0.13 c	2.2 ± 0.12 d	3.4 ± 0.01 a	2.1 ± 0.14 d	3.3 ± 0.11 ba	2.9 ± 0.17 bc
GA_{20}	0.9 ± 0.15 c	1.5 ± 0.17 b	1.3 ± 0.12 cb	1.3 ± 0.07 cb	1.4 ± 0.15 cb	2.9 ± 0.16 a
GA_{24}	4.7 ± 0.27 e	6.2 ± 0.13 c	8.9 ± 0.18 a	7.5 ± 0.05 b	7.6 ± 0.08 b	5.3 ± 0.01 d
GA_{44}	1.7 ± 0.12 a	1.6 ± 0.16 a	0.9 ± 0.13 b	0.5 ± 0.13 b	1.5 ± 0.06 a	1.7 ± 0.12 a
GA_{53}	0.8 ± 0.10 b	1.1 ± 0.14 b	ND	0.2 ± 0.12 c	1.7 ± 0.15 a	1.1 ± 0.10 b
Total	37.4	59.6	51.3	49.0	60.8	58.2

The data is the mean of the triplicate experiments. GAs amounts are calculated by GC-MS quantification using deuterated internal standards. ND, not determined. All values are mean ± SEM, N=3; Values with different characters are significantly different at $P<0.05$.

(Table 1).

Thus, *B. cereus* MJ-1, *B. macroides* CJ-29 and *B. pumilus* CJ-69 were determined to be plant-growth-prompting rhizobacteria (PGPR).

GAs biosynthetic pathways in red pepper seedlings

Nothing is known about the biosynthesis pathway of GAs in the vegetative and reproductive tissues of the red pepper. To date, the following GAs have been detected in the cell-free culture broth of red pepper rhizobacteria: GA_1, GA_3, GA_4, GA_5, GA_7, GA_8, GA_9, GA_{12}, GA_{19}, GA_{20}, GA_{24}, GA_{34}, GA_{36}, GA_{44}, and GA_{53} (Joo *et al.*, 2004). In order to check authentic GAs in the seedling shoots of red peppers, the deuterated GAs, KRI values, retention times, and parent ions from each of the HPLC fractions from seedlings were evaluated by GC-MS selected ion monitoring. In general, two major biosynthesis pathways of physiologically active GAs appear to operate in vegetative shoots of most higher plants, depending on the species and tissue analyzed (MacMillan, 1997; Kim *et al.*, 2003). The multistep conversion of GA_{12}-aldehyde, which is formed from *ent*-kaurene, into active GAs proceeds via either the early C-13 hydroxylation pathway (GA_{12}-aldehyde, GA_{12}, GA_{53}, GA_{44}, GA_{19}, GA_{20}, GA_1) or the early non C-13 hydroxylation pathway (GA_{12}-aldehyde, GA_{12}, GA_{15}, GA_{24}, GA_9, and GA_4) (Hedden, 1997). The GAs produced by the early C-13 hydroxylation pathway and the early non C-13 hydroxylation pathway, as well as some other GAs, were detected the first time in red peppers (Table 2). The total content of the GAs of the non C-13 hydroxylation pathway was about twice that of the GAs of the early C-13 hydroxylation pathway. Thus, the major pathway of endogenous GA metabolism in the seedling shoots of red peppers is likely to be the non-C-13 hydroxylation pathway, although the early C-13 hydroxylation biosynthetic pathway is also presented (Fig. 1).

Increment of endogenous GAs content of red pepper seedlings by GAs producing PGPR

It was previously reported that *B. cereus* MJ-1, *B. macroides* CJ-29 and *B. pumilus* CJ-69 produced GAs (GA_1, GA_3, GA_4, GA_5, GA_7, GA_9, GA_{12}, GA_{19}, GA_{20}, GA_{24}, GA_{34}, GA_{36}, GA_{44}, and GA_{53}) and that the relative content of the 3β-hydroxylated GAs (GA_1, GA_3, GA_4, and GA_{36}) was higher than that of other GAs in the culture broth of the PGPR (Joo *et al.*, 2004). A few GAs were identified in the control (non-inoculated) medium but the content was very low compared with the GAs produced in the presence of rhizobacteria (data not shown). Among the GAs found in the bacteria, GA_{15} was not observed (Joo *et al.*, 2004). However, GA_{15} was observed in the red pepper shoot tissue, implying that the GA biosynthesis pathways in the red pepper shoot are different from those in the PGPR.

In the red pepper shoot tissue, the total endogenous GAs content increased 1.59-fold over control levels with the inoculation of *B. cereus* MJ-1 (Table 2). In the culture broth of *B. cereus* MJ-1, four growth-active GAs (GA_1, GA_3, GA_4, and GA_7) showed higher levels than other precursors GAs (Joo *et al.*, 2004). These four physiologically active GAs (GA_1, GA_3, GA_4, and GA_7) may play an important role in regulating growth in higher plants. All

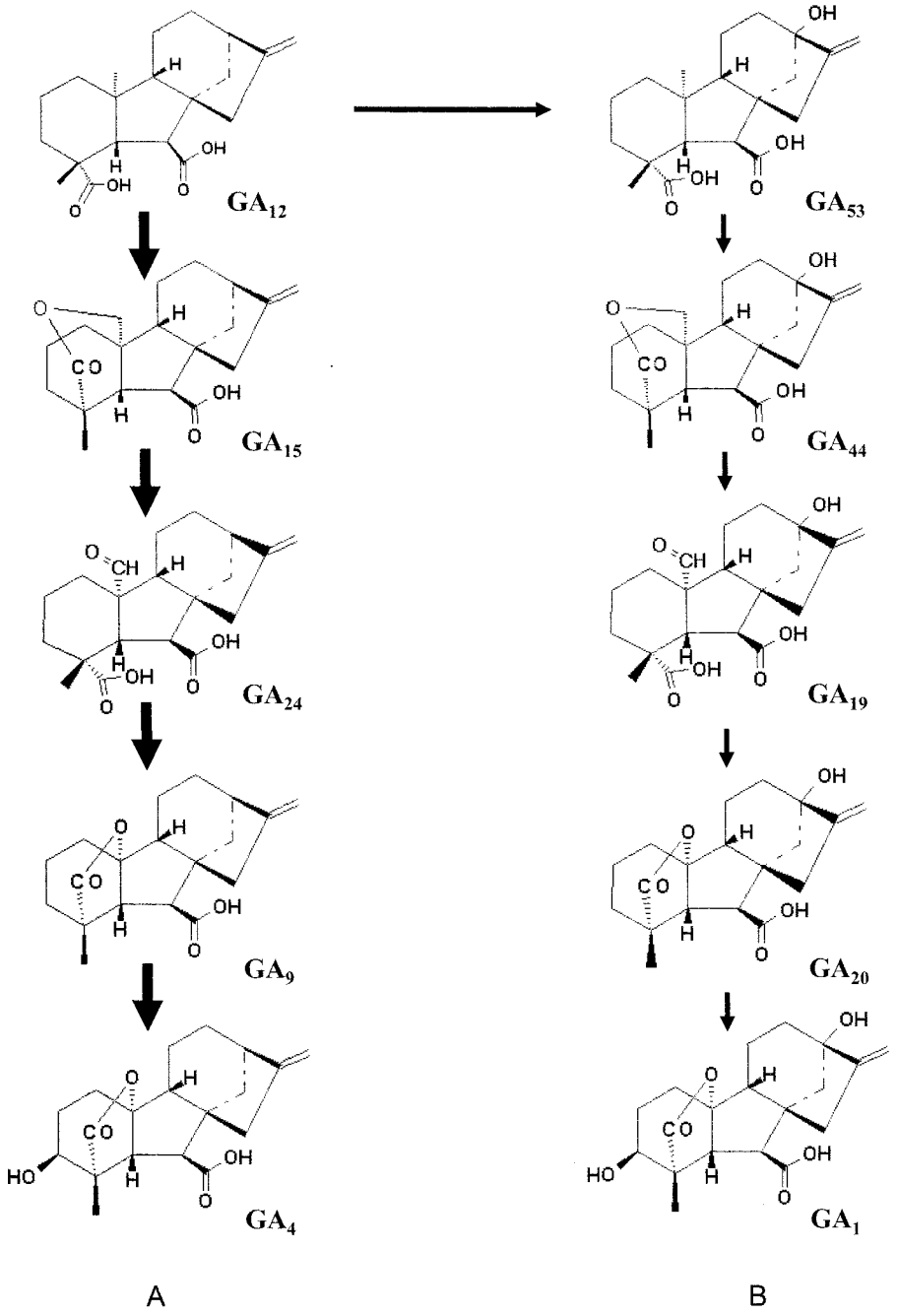

Fig. 1. Non C-13 hydroxylation pathway (A) and the early C-13 hydroxylation pathway (B) routes (Heddden, 1997). Two gibberellins (GAs) biosynthesis pathways in red pepper plants.

four were also identified in the red pepper shoots (Table 2). In the red pepper shoots inoculated with *B. cereus* MJ-1, levels of growth-active GAs and precursor GAs (GA_9 and GA_{20}) were also 1.3 to 1.4 times higher than in the non-inoculated control (Table 2). Among the endogenous GAs, the content of GA_{12} increased 43.0-fold and GA_{15} increased 5.5-fold upon inoculation of *B. cereus* MJ-1 (Table 2). We speculate that the content of GA_{12} and GA_{15}, early GAs in the non-C-13 hydroxylation pathway, were dramatically increased by bacterial inoculation. In addition, the content of GA_{12} and GA_{15} was also increased by inoculation with *B. macroides* CJ-29 and *B. pumilus* CJ-69. The content of GA_{44} and GA_{53}, GAs of the early C-13 hydroxylation pathway, however, tended to decrease with the addition of *B. macroides* CJ-29 and *B. pumilus* CJ-69.

The total content of the endogenous GAs and the four growth-active GAs was also increased by inoculation with *B. macroides* CJ-29 and *B. pumilus* CJ-69. However, the increment was less than that of each inoculated alone or

co-inoculated with *B. cereus* MJ-1 (Table 2). When strains CJ-29 and CJ-69 were inoculated together, no significant increase was observed in endogenous GAs, or in the four active GAs over values resulting from inoculation with either CJ-29 or CJ-69 alone (data not shown).

Based on the above results, we speculate that the endogenous GAs levels of red pepper shoots results at least partially from increased uptake of GAs produced by the PGPR. It also appears that the plant-growth promotion originated from the growth-active GAs: GA_1, GA_3, GA_4, and GA_7. These GAs may have had their origin through the plants' GAs biosynthetic pathway, or via uptake as either active GAs or their precursors.

Root colonization on red pepper roots

The GAs content was comparatively higher in inoculated plants than that in non-treated controls (Table 2). However, significant differences in bacterial colonization were observed among rhizobacteria species (Fig. 2). Although all three strains were able to colonize the red pepper root system, *B. cereus* MJ-1 was most effective in colonizing the red pepper rhizosphere. The density of *B. cereus* MJ-1 was higher in plants that were inoculated only with *B. cereus* MJ-1, than in plants inoculated with *B. macroides* CJ-29, *B. pumilus* CJ-69, or MJ-1 in combination with either of these rhizobacterial strains (Fig. 2).

When inoculated with *B. cereus* MJ-1 or any combination of bacteria including MJ-1, the fresh weights of shoots and roots were a little higher than those of plants inoculated with *B. macroides* CJ-29 or *B. pumilus* CJ-69 (Table 1). The endogenous active GA content was also a little higher in plants inoculated with bacterial mixes that include MJ-1 than that in plants inoculated with *B. macroides* CJ-29 or *B. pumilus* CJ-69 alone (Table 2). These

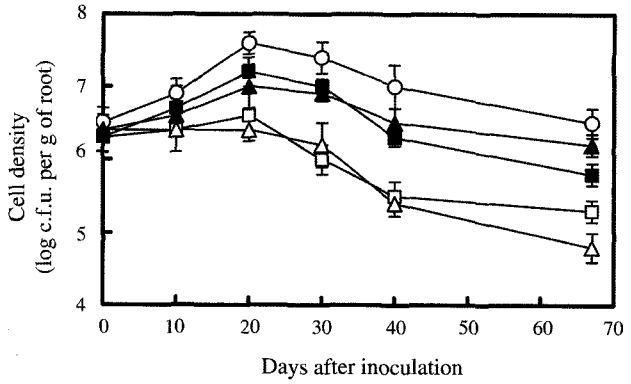

Fig. 2. Population density of rhizobacteria on the root systems of red pepper plug seedlings. Each red pepper plug seedling, which was planted in 40 g of wet potting soil, was treated with 1 ml of bacteria diluted with tap water to an estimated cell density of 10^8 c.f.u. ml^{-1}. When mixed bacterial treatment was used, the mixture ratio was 1:1 (v/v). The data is the mean of six individual experiments. ○-○, *Bacillus cereus* MJ-1; □-□, *B. macroides* CJ-29; △-△, *B. pumilus* CJ-69; ■-■, MJ-1 + CJ-29; ▲-▲, MJ-1 + CJ-69.

results support the idea that the promotion of plant growth by the PGPR producing GAs is positively related not only to the endogenous GA content of the plant shoot tissue, but also to the effectiveness of root colonization by the bacteria. Rhizobacteria that are capable of colonizing efficiently in roots are much more effective in plant growth promotion (Weller, 1988). Of course, increased colonization should also increase the likelihood of increased uptake of rhizobacteria-secreted GAs.

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